FINAL REPORT

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DEVELOPMENT OF GENETIC TRANSFORMATION TECHNOLOGY FOR COMMERCIAL CULTIVARS OF WHEAT AND BARLEY

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Development of Genetic Transformation Technology for Commercial Cultivars of Wheat and Barley

April 1998 to March 2002

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ABSTRACT

The main objective of this project has been to optimize the three important factors; tissue culture, gene constructs and gene delivery techniques, for improvement of transformation techniques for wheat and barley cultivars grown in Saskatchewan. Plant regeneration experiments with wheat and barley identified explants optimal explants for transformation of different classes of wheat and barley. A mature embryo regeneration system that provides an easy and fast way of obtaining explant material for transformation has been developed. The mature embryos, when induced to form multiple shoots by treatment with an unclassified growth hormone, has been found to be superior to immature explants for regeneration of several classes of wheat and barley. This system is of special value for winter wheat, which requires four to five months of growth and cold-treatment before immature spikes can be harvested. Twenty different gene constructs with selectable marker, scorable marker or anti-sense constructs gene have been assembled and used in transformation experiments of both wheat and barley. Possibilities to produce transgenic wheat and barley free from herbicide- or antibiotic-resistance genes has been provided by assembly of a dual T-DNA cassette vector to be used in *Agrobacterium* transformation experiments. About 600 each of putatively transformed wheat and barley plants have been regenerated from transformation experiments using particle bombardment. Among these plants, stable integration of transgenes has been confirmed for wheat plants transformed with a neomycin phosphotransferase (*nptII*) gene, a Fusarium resistance gene and various anti-sense starch branching enzyme genes. Transgenic barley plants produced carry the BAR gene encoding resistance to phosphinotricin acetyl transferase (PAT), an active ingredient of herbicides Basta and Liberty Link. Most transgenic plants are healthy, show normal seed set and Mendelian inheritance of transgenes. However, a field trial with transgenic barley revealed problems with gene silencing and variable thousand-kernel weights among different transgenic lines. Delayed heading and altered starch granule size distribution was noted among some anti-sense starch branching enzyme plants, the latter effect is possibly due to the lower level of branching enzyme activity during endosperm

development. Transformation experiments with wheat and barley explants using *Agrobacterium* has provided evidence of gene transfer to host genome, but recovery of transgenic plants has been difficult. The main problem has been elimination of the *Agrobacterium* strain after infection.

EXECUTIVE SUMMARY

The molecular, biochemical and tissue culture work of the project was done in the Cereal Biotechnology Group, Plant Biotechnology Institute, under supervision of Dr. Ravindra Chibbar. Field trials were conducted at the Kernen Crop Research Farm, University of Saskatchewan under supervision of Dr. Brian Rossnagel, Crop Development Centre.

During the 46 month's duration of the project, a total of eight people were hired on a full-time basis as listed below. The personnel was made up of a technical assistant (TO1 level) for 36 months, two technical assistants (TO2) for 44 and 35 months, two Post-doctoral Fellows for 24 and 36 months each and one Research Officer for 46 months.

Dr. Ravindra Chibbar – Principal Investigator 15%.

Dr. Monica Båga – Project Manager - Research Officer 100%, May 20 1998 until March 28 2002.

Dr. Hao-Jie Chen – NSERC Post-doctoral Fellow 100%, Jan 11 1999 until Dec 31 2000.

Dr. Ganeshan Seedhabadee – NSERC Post-doctoral Fellow 100%, Jan 6 1999 until Dec 31 2001.

Mr. Gregory Peterson - Technical Officer II 100%, Aug 10 1998 until April 30 1999.

Ms. Maureen Troesch – Technical Officer II 100%, July 6 1998 until March 28 2002.

Ms. Melanie Dauk - Technical Officer II 100%, May 17 1999 until May 16 2000.

Mr. Cecil Granat - Technical Officer I 100%, July 2 1998 until March 31 2001.

Mr. Chandra Rathnayaka – Technical Officer II 100% June 15 2000 until May 15 2001.

Milestones 1 was done according to schedule, with the exception for vectors carrying SAR/MAR sequences. The SAR/MAR constructs were omitted due to recent data questioning the benefit of these sequences in monocot systems as discussed on page 23. Milestones 2 and 3 and 4 were conducted according to schedule. Extra effort was made to optimize the mature embryo regeneration system, primarily for barley cultivars (Milestone 3). Transformation experiments using particle bombardment and regeneration of transgenic wheat and barley lines was done according to schedule (Milestones 5 and 6). *Agrobacterium*-mediated transformation, as outlined in Milestones 5 and 6, were done with both wheat and barley cultivars using different explant tissues. However, these experiments only resulted in a few putatively transformed plants due to problems with elimination of *Agrobacterium* after infection. So far, none of the plants obtained from *Agrobacterium*-mediated transformation appear to carry transgenes. Thus, a comparison of transgenic plants produced with biolistics or *Agrobacterium*-mediated gene delivery could not be done as planned in Milestone 8. All molecular and biochemical analyses of wheat and barley transgenics obtained from particle bombardment are presented in Milestone 7. A field trial of barley transgenic lines was done according to Milestone 9.

RESEARCH SUMMARY

Milestones.

1. Construction of monocot expression vectors containing selectable and scorable marker genes and/or SAR and MAR sequences.
2. Comparison of regeneration response of different tissue explants from four wheat genetypes (winter, CPS, HRS and durum).
3. Comparison of regeneration response of different tissue explants from four barley genotypes (malting, feed, hulless and hulled.
4. Construction of super-binary vectors containing expression cassettes in Milestone 1 and engineering of *Agrobacterium* strains to harbor super-binary vectors.

5. Genetic transformation of selected wheat genotypes with biolistics and *Agrobacterium* mediated gene delivery.
6. Genetic transformation of selected barley genotypes with biolistics and *Agrobacterium* mediated gene delivery.
7. Greenhouse evaluation of wheat and barley transgenics.
8. Molecular/cytogenetic characterization of wheat and barley transgenics to compare integration of inserted genes into host genomes produced with biolistics or *Agrobacterium*-mediated gene delivery.
9. Field-testing of wheat and barley transgenics.

Results achieved.

Milestone 1. A total of nine vectors encoding optimal expression of selectable or scorable marker genes in wheat and barley tissues have been assembled. The selection vectors provide resistance to the antibiotics Geneticin or Paramomycin or the herbicide Liberty Link and have been used in most of our transformation experiments. To allow detection of transformed cells and tissues by simple assays, a set of expression cassettes directing production of the marker enzymes luciferase, green fluorescent protein (GFP) and β-glucoronidase (GUS), respectively, have been made. Two of the constructs used in this project encode hybrid proteins with both selectable marker and scorable marker properties (*nptII::uidA*; *bar::uidA*). Twelve constructs encoding fragments of different isoforms of starch branching enzyme (SBEI, SBEIIa, SBEIIb) have been assembled and used in various transformation experiments. Ten of the anti-sense expression cassettes are under the control of the constitutive rice actin promoter, whereas two are controlled by an endosperm-specific promoter. In addition, we have assembled a hairpin cloning vector for construction of efficient anti-sense constructs functional in cereal cells as described by Smith et al. (2000). A vector encoding Fusarium-resistance has been obtained and used in transformation experiments. Constructs containing SAR/MAR sequences were not made due to doubts about their benefits in monocot systems as discussed on page 23.

Milestone 2. Five different tissues of wheat; scutella, immature embryos, inflorescences, leaf bases and mature embryos of different classes of wheat were tested in regeneration experiments. Isolated scutella provided good regeneration for soft white spring wheat and durum wheat, but scutella isolated from winter and hard red spring cultivars showed low regeneration frequencies. Immature inflorescence was the optimal immature explant tissue found for Canadian Prairie Spring (CPS) and hard red spring wheat, although the number of shoots formed per explant of hard red spring wheat was low. Through modifications of the inflorescence regeneration media, the regeneration frequencies were increased three-fold. Leaf bases and mature embryos as explants were primarily tested with winter wheat and provided better and faster regeneration than immature tissues, such as inflorescences, scutella or leaf bases. The mature embryo system is based on regeneration of shoots directly from embryos of mature seeds, an explant source that is always available. Regeneration of plants from mature embryos was found to be of special value for winter wheat, which requires four to five months of growth and cold-treatment before immature explants can be isolated. The regeneration rate from mature embryos was improved by inclusion of an unclassified growth hormone (UGR) in the regeneration media, which induced formation of multiple shoots directly from the explant tissue without a callus interphase.

Milestone 3. The regeneration frequencies for callus produced from immature scutella, leaf bases and mature embryos of four barley genotypes; CDC Dawn, CDC McGwire (feed), CDC Copeland (2-row malting) and BT459 (6-row malting) were compared. Regeneration of plants from callus derived from isolated immature scutella and leaf-bases was improved through modifications to the media. A maximum of four shoots per explant was obtained. In contrast to calli produced from scutella and leaf-bases, callus derived from mature embryos was found to be of poor quality for regeneration. However, through inclusion of the cytokinin N^6Benzyladenine (BAP) into the regeneration media, mature embryos, as well as leaf-bases, could be induced to form multiple shoots without passing through a callus phase. This induction was improved by inclusion of an unclassified growth hormone (UGR) into the media to give up to eight shoots per leaf-base. Mature

embryos were found to be an even better explant source than leaf-bases for multiple shoot induction and gave between eight to 14 shoots per explant when tested with the four barley cultivars. Furthermore, the total number of shoots per explants can be further increased by prolonged incubation of explant on the regeneration media. From these experiments we concluded that the mature embryos system of barley, when induced to form multiple shoots, is superior to immature explant material for plant regeneration. Since production of mature embryos does not require constant growth of donor plants, we have obtained an easier, faster and cheaper way of obtaining explant material for transformation. The mature embryo regeneration system has also been found to be useful for wheat (Milestone 2) rye and oat.

Milestone 4. Two co-integrate vectors have been assembled and used in *Agrobacterium*-mediated transformation of wheat and barley. These vectors express NTPII (pSBIII-KA construct) and BAR (pSBIII-UB construct) providing antibiotic resistance and herbicide resistance, respectively. To allow removal of inserted genes encoding antibiotic or herbicide resistance from generated transgenic lines, we have assembled three vectors that can carry two separate T-DNA cassettes. The two T-DNA cassettes will be inserted with high frequency into different parts of the host genome upon infection, and thus, integrated transgenes can be separated in the following generations. One of the T-DNA cassettes carries a selectable (NPTII or BAR gene) or a GUS scorable marker gene. Second T-DNA cassette will carry the gene of interest.

Milestone 5. In total, 608 putative transgenic lines belonging to five different classes of wheat (soft white spring, CPS, durum, red winter and hard red spring) have been regenerated. All of these lines were obtained through particle bombardment. Soft white spring wheat, which is easiest to transform among the wheat classes, produced 340 putatively transformed lines, all derived from scutella explants. Isolated scutella was also the preferred explant for transformation of durum wheat, for which six putative transgenic plants were regenerated. A total of 124 regenerated winter wheat plants that were regenerated from bombardment of isolated scutella and immature embryos with

anti-sense *Sbe1* or *Sbe2* constructs. Inflorescence explants were used in transformation experiments with the CPS cultivar AC Karma and hard red spring wheat line BW720. Most of these regenerated plants are putatively transformed with anti-sense *Sbe1* or *Sbe2* constructs. Transformation experiments using *Agrobacterium tumefaciens* strains for gene delivery and AC Karma (CPS) inflorescence or AC Nanda (soft white spring) scutella as explants have not produced any confirmed transgenic lines. Although there is evidence of gene delivery into host genome, the regeneration of plants from transformed tissues has been difficult. The main problem lies with elimination of *Agrobacterium* after infection, without causing negative effects on plant regeneration.

Milestone 6. The barley cultivars used in our study were found to show great variation in their resistance to L-PPT, the agent used for selection of plants expressing the BAR gene. Therefore, the optimal L-PPT concentration for regeneration of scutella for three cultivars was determined. We also compared effects of the bombardment procedure on regeneration efficacy of different barley explants. For bombarded scutella, an average regeneration frequency of 1.8% was obtained, whereas leaf-bases and mature embryos showed about eight times higher regeneration frequencies (15.9% and 13.9%, respectively). Thus, it appeared that regeneration from bombarded scutella is rather inefficient in comparison to leaf-bases and mature embryos. A total of 586 regenerated plants were obtained from bombardment of scutella, leaf-bases and mature embryos of both malting and feed genotypes. Most of these regenerated plants were from experiments using a construct encoding Fusarium resistance. Many transformation experiments using *Agrobacterium* and barley scutella, leaf-bases or mature embryos have been performed. However, similar to *Agrobacterium*-mediated transformation of wheat, plant regeneration from host cells carrying inserted T-DNA has been difficult. A set of different factors of importance for *Agrobacterium*-mediated transformation were tested to improve infection, elimination of bacteria after infection and regeneration. These factors included the effective concentration of antibiotics and acetosyringone, effect of co-cultivation time, cell density of *Agrobacterium* and pre-treatment of *Agrobacterium* incubation medium. After optimization, a few barley barley plants have been regenerated

from transformation experiments with *Agrobacterium*. However, we have so far been unable to confirm presence of transgenes in these lines.

Milestone 7. Wheat lines confirmed to be transgenic by molecular analysis have all been obtained from particle bombardment of CPS inflorescence and scutella from soft white spring and durum wheat. Experiment with the highest transformation frequency was recorded for Karma inflorescence (4.0%), whereas 2.8% was the best result obtained from Fielder scutella. Analysis of six durum lines showed that five carried the selectable marker gene, pBARGUS, and two of these also carried the Fusarium head blight-resistance gene. Four of the BAR-positive lines were confirmed to be resistant to L-PPT when tested by leaf paste assay. So far, nine Fielder and eight Karma lines have been confirmed positive for different anti-sense *Sbe2* constructs by PCR analysis. A more detailed molecular and biochemical analysis has been done on Fielder (soft white spring) plants generated from two experiments with Fielder scutella and the pKA8 and anti-sense *Sbe1c* constructs. Out of 27 analyzed lines, 19 were shown to carry the selectable marker gene and twelve of these lines also carried the anti-sense *Sbe1c* construct. Southern blot analysis of progeny from 12 of the transformed lines showed that the both transgenes were co-inherited and showed Mendelian segregation in 11 of the lines. The different transgenic lines showed some variation in date of anthesis, which was not correlated to the presence of transgenes for all lines. In addition, a difference in the ratio of small and large starch granules, as compared to the parent line, was found for some of the transgenic lines. The altered starch granule distribution is likely an effect of anti-sense expression of *Sbe1c*. Growth of transgenic lines in two different growth rooms revealed that growth conditions had great influence on large/small starch granule size ratio.

Milestone 8. A comparison of transgenic lines obtained through particle bombardment and *Agrobacterium*-mediated transformation was not done, due to lack of confirmed *Agrobacterium* transformed wheat and barley lines. Analysis data of wheat and barley transgenic lines obtained from particle bombardment are presented in Milestones 6 and 7.

Milestone 9. Progeny of seven transgenic barley lines carrying the BAR gene encoding resistance to the herbicide Liberty Link were field-tested during the 2000-growing season. Spray test of plants at the three-leaf stage did not reveal any significant resistance to the herbicide, even when low (0.8 L/acre) herbicide concentration was used. Nor did the plants express any significant BAR activity as measured by the phosphinotricin acetyl transferase (PAT) assay. However, the majority of the tested plants were found to carry the BAR transgene according to PCR analyses. Thus, gene silencing of the BAR gene was likely the reason for lack of PAT activity and herbicide resistance in the transgenic lines. Most of field-tested plants showed normal heading date, height and maturity, although some variation was noted in 1000-kernel weigh between the individual lines when compared to the parent lines or non-tranformed regenerated lines.

RESEARCH OUTPUTS

Intellectual Property

Ganeshan S, Båga M, Rossnagel BG, Harvey BL, Hucl PJ, Fowler DB, Fox SL, Scoles GJ and Chibbar RN (2001). An efficient *in vitro* regeneration system for cereals. Provisional patent filed on May 02, 2001 # 60/287,731.

Research Publication*

Ganeshan S, Båga M, Harvey BL, Rossnagel BG, Scoles GJ and Chibbar RN. (2003). Efficient production of multiple shoots from thidiazuron-treated mature embryos and leaf-base/apical meristems of barley (*Hordeum vulgare* L.) genotypes. Plant Cell Tissue and Organ Culture (in press).

* *Several experiments are currently in progress, and near completion. As and when these results will be published in the near future, Agri-Food Innovation Fund will be credited for support of the research reported in the respective publications.*

Conference Presentations

Platform Presentations (Oral)

Båga, M and Chibbar RN (1999). Principles and practices for transgenic cereal production. A symposium "Improvement of cereal production through biotechnology" American Association of Cereal Chemists, Annual meeting, Seattle, Wa, USA October 31-November 3, 1999.

Båga M (2001) Modification of Starch in Cereals: The Development of Novel Nutraceuticals. Saskatchewan Nutraceutical Network (SNN), Saskatoon, Sask. Jan. 25, 2001.

Chibbar, RN (2002). Cereal grains biotechnology: Opportunities and challenges. Canada Grains Council, Annual Meeting, Session: Regulatory Change for Future Growth. Winnipeg, April 7-9, 2002.

Chibbar, RN (2002) Barley improvement through biotechnology: Opportunities and Challenges, Western Barley Growers Association 25th annual convention, Calgary, Alberta, Canada, February 13-15, 2002.

Chibbar, RN, Båga, M, Davis, JP and Hucl P (2001) Wheat starch biosynthesis and modification. Starch Round Table October 11-13, 2001, Charlotte, North Carolina. (Keynote lecture in the session Advances in Starch Synthesis and Biotechnology.).

Chibbar, RN (2001) Discussion Moderator "Biotechnology and the Grain Industry: Opportunities and Challenges". American Association of Cereal Chemists, Annual Meeting, Charlotte, NC, USA, October 14-18, 2001.

Chibbar, RN, Båga, M, Davis, JP and Hucl, P. Wheat improvement through Biotechnology. Second International Wheat Quality Conference, Manhattan, Kansas, May 19-23, 2001.

Chibbar, RN (2001) Cereal Biotechnology – An introduction. Biotechnology Round Table, organized by Grocery Headquarters, Epcot Centre, Orlando, Florida, USA, February 5-6, 2001.

Chibbar, RN (2001) Small grains cereal molecular biology: opportunities and challenges. Montana Ag Business Association, Annual Convention, Great Falls, Montana, Jan 24 – 26, 2001.

Ganeshan S, Båga M and Chibbar RN (2001) Genetic transformation systems for barley (*Hordeum vulgare* L.) genotypes of commercial value. Plant Biotechnology Institute Symposium, National Research Council Canada, Saskatoon, Saskatchewan, February, 2001.

Ganeshan S, Båga M, Harvey BL, Rossnagel BG, Scoles GJ and Chibbar RN (2001) In vitro regeneration from mature embryo explants of barley (*Hordeum vulgare* L.). Fifth Canadian Plant Tissue Culture and Genetic Engineering Workshop, International Association for Plant Tissue Culture and Biotechnology (Canadian Section), Saskatoon, Saskatchewan, May 12-15, 2001.

Poster Presentations

Ganeshan S, Båga M, Harvey B, Rossnagel B, Scoles GJ and Chibbar RN (2002). Use of mature embryo explants for regeneration of barley (*Hordeum vulgare* L.). 10th IAPTC&B Congress Plant Biotechnology 2002 and Beyond A celebration and showcase. Disney's Coronado Spring Resort, Florida, USA June 23-28, 2002.

Ganeshan S, Caswell K, Leung N, Båga M, Harris L, Gleddie S, Fowler DB, Harvey BL, Hucl P, Rossnagel BG, Scoles GJ and Chibbar RN (2001) A transgenic approach to confer resistance to fusarium head blight in barley and wheat. Poster presented

at the Canadian Workshop on Fusarium Head Blight, Ottawa, Ontario, November 3-5, 2001.

Ganeshan S, Troesch M, Båga M, Rossnagel B, Harvey B, Scoles GJ and Chibbar RN (2000) Genetic transformation of barley (*Hordeum vulgare* L) using microprojectile bombardment and *Agrobacterium tumefasciens*. Sixth International Society for Plant Molecular Biology Conference, Quebec City, PQ, Canada June 18 – 24, 2000.

Seminar Presentations

Båga M (2001) Genetic Engineering of Wheat Starch Quality. Dept. of Plant Biology, Swedish University of Agricultural Sciences, Uppsala, Sweden. Dec. 10,2001.

Båga M (2001) Starch - A Target for Genetic Engineering of Wheat. Dept. of Biochemistry, Univ. of Saskatchewan, Saskatoon, Sask, March 12, 2001.

Båga M (2000) Starch - A Target for Genetic Engineering of Wheat. Plant Biotechnology Institute, Saskatoon, Sask. Nov. 22, 2000.

Chibbar RN (2002) Genetic modification of starch structure in wheat. Western Regional Research Centre, USDA/ARS, Albany, California, USA. January 18, 2002.

Chibbar RN (2001) Isolation and characterization of a novel starch branching enzyme preferentially associated with large A-type wheat starch granules. Graduate program in plant cell and molecular biology. University of Florida, Gainesville, FL, USA. February 07, 2001.

Chibbar RN (2001) Starch a target for genetic modification in wheat. Department of Plant Sciences, Montana State University, Bozeman, Montana, USA. January 25, 2001.

Chibbar RN (2000) Genetic modification of starch granule proteins to alter starch structure in wheat. Consumer and Nutrition Sector, Monsanto Company, St. Louis, USA, January 28, 2000.

Chibbar RN (1999) Genetic modification of starch structure in wheat. Agritope Inc. Portland, Oregon, USA. October 29, 1999.

Chibbar RN (1999) Genetic engineering of wheat for Fusarium resistance. Semi-Arid Prairie Agricultural Research Centre, Swift Current, Sask. Canada, May 21, 1999.

Awards and Honors

Dr. S. Ganeshan, a post-doctoral fellow in the project was awarded a Young Scientist Fellowship (US$ 1,000), by the International Association for Plant Tissue Culture and Biotechnology (IAPTC&B), Gainesville, Florida, USA to present a poster at the 10th IAPTC&B Congress, "Plant Biotechnology 2002 and beyond: A celebration and showcase". Disney's Coronado Spring Resort, Florida, USA June 23-28, 2002.

PROJECT OBJECTIVES

Genetic transformation of plants is a complicated process, which requires optimal performance of several technologies in order to produce a transgenic line (Fig. 1). Most transformation protocols developed for cereals are optimized for model cultivars, which are not used for commercial grain production. Therefore, produced transgenic lines may not show the desired agronomic performance and added value conferred by the transgene may not compensate for the negative attributes of the line. To improve the value of produced transgenic lines, inserted transgene(s) have been transferred to commercial lines by breeding. This extra step adds both time and cost to production of commercial transgenic cultivars. To speed up production of transgenic wheat and barley, the main objective of this project is to modify or develop new technologies to directly transform cultivars of importance to Saskatchewan producers.

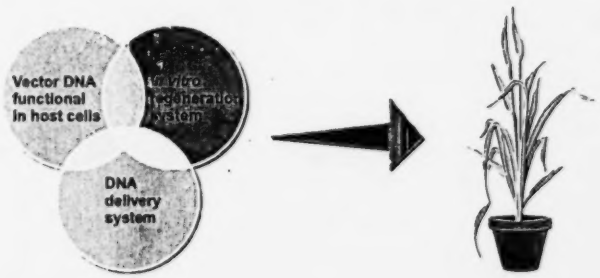


Fig. 1 Production of transgenic plants requires interaction of several technologies.

The most important factor in a transformation process is the tissue culture system that will allow regeneration of plants in large quantities from donor tissues. Factors such as choice of explant, physiological condition of explant, culture conditions, medium composition and genotype will determine the overall success of the tissue culture. The explants are obtained from immature tissues, but only a few tissue types are suited for *in vitro* culture. The most commonly used tissues for regeneration and transformation of wheat and barley are immature embryos, scutella isolated from immature embryos and

inflorescences (Fig. 2). For these explants, growth conditions of the donor plants and the age of the explant are very critical for the overall success of the subsequent regeneration steps. However, it is often found that explant tissues of commercial cultivars respond poorly in tissue culture, and wheat and barley cultivars grown in Saskatchewan are no exceptions.

Another important factor to consider is the design of DNA constructs to be introduced into the host cells. It is imperative that the expression cassettes are properly recognized by the host cell transcriptional and translational machineries to efficiently encode biosynthesis of transgene products. In general, gene regulatory elements isolated from monocot species perform best in cereals such as wheat and barley. To efficiently deliver the gene constructs to the nuclei of host

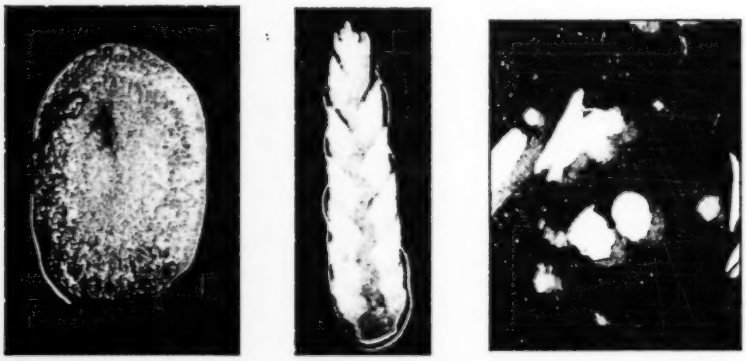


Fig. 2. Explant tissues commonly used for regeneration of wheat and barley. From left to right are shown isolated scutella, immature inflorescence and immature anthers.

cells, the method of DNA delivery has to be properly chosen without compromising the efficiency of the tissue culture system. So far, only particle bombardment has shown repeated success for transformation of wheat and barley cultivars that respond well in tissue culture. Only a few transgenic wheat and barley lines have been reported using *Agrobacterium*-mediated transformation techniques (Tingay et al. 1997; Cheng et al. 1997). However, the reported transformation frequencies using either DNA delivery technique are low and needs to be improved for large-scale production of transgenic lines. In order to select for transformed shoots and plantlets, the selective agent and

selection strategy used during the regeneration step have to be carefully chosen and optimized. Presence of transgenes and analysis their expression pattern in putatively transgenic lines needs to be confirmed by biochemical and molecular assays. These tests are also done on progeny of primary transformants and subsequent generations to ensure that the inserted transgene(s) do not undergo gene rearrangements and are inherited in a predictable manner. Finally, the agronomic performance of transgenic plants needs to be assessed by field trials to confirm that the transformation procedure has not negatively affected the overall performance of the crop plant.

TECHNICAL REPORT

Milestone 1: Construction of monocot expression vectors containing selectable and scoreable marker genes and/or SAR and MAR sequences.

Basic cloning vectors.

Two cloning vectors based on the rice actin promoter and the transcriptional termination sequence from the nopaline synthase gene have been assembled (Fig. 3).

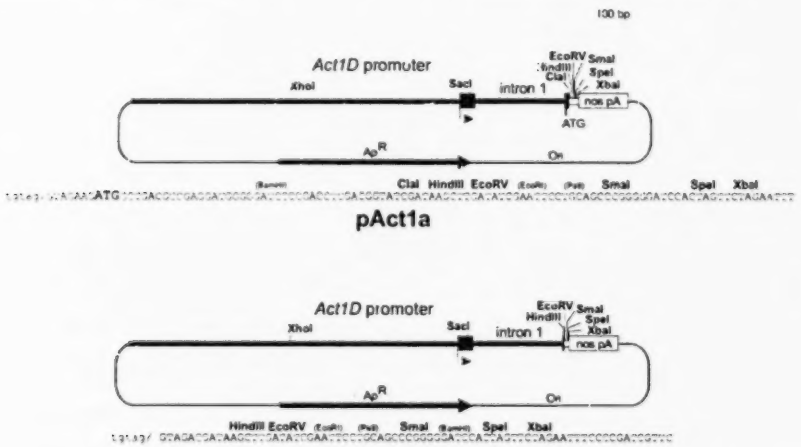


Fig. 3. Schematic illustration of cloning vectors pAct1a and pAct1c.

The pAct1a vector contains an ATG translational start codon in front of the multiple cloning site, whereas pAct1c lacks the start codon. These vectors allow construction of

both transcriptional and translational fusions and have been used to assemble several expression constructs presented in this report.

The cloning vector pKA9 (Fig. 4) was assembled by linking the dual starch branching enzyme promoter isolated from wheat (Båga et al. patent) with the nopaline synthase polyadenylation sequence. This construct allows constitutive expression of genes in cereal tissues when gene of interest is inserted into the unique XhoI and SacI sites.

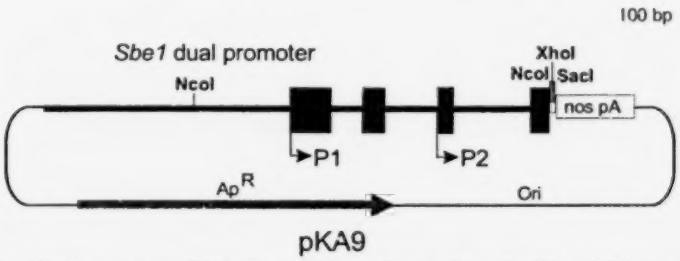


Fig. 4. Cloning vector pKA9. Start of transcription from the P1 and P2 promoters are indicated. *Sbe1* exon sequences are represented by black boxes.

Monocot transformation vectors carrying selectable and scorable marker genes.

To identify transgenic shoots during the regeneration step, an expression cassette carrying a selectable marker gene is introduced together with gene of interest during transformation. In general, the selectable marker encodes resistance to an antibiotic or herbicide, which is added to the regeneration medium. Thus, by using a highly active promoter to drive expression of the selectable marker gene, transgenic shoots will survive on the selective medium and the vast majority of non-transgenic cells and tissues will be eliminated. The selectable marker gene and gene of interest may be located on the same plasmid or on separate plasmids. Because the co-transformation frequencies for co-introduced plasmids often exceeds 50% (e.g. Barro et al. 1997; Stoger et al. 1998), we have chosen to separate the selectable marker gene and gene of interest onto different plasmids. This has simplified the construction of expression vectors and allowed more combinations of selectable marker and gene of interest to be used.

The rice actin promoter including its first intron is highly active in most monocot

tissues (McElroy et al. 1991) and was used for construction of the selection vector, pKA8 (Fig. 5). This vector carries *nptII* encoding neomycin phosphotransferase (NPTII), that

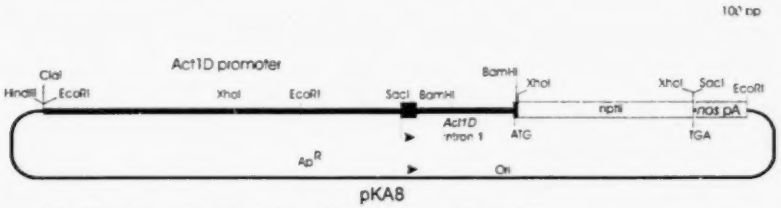


Fig. 5. Schematic illustration of selection vector pKA8.

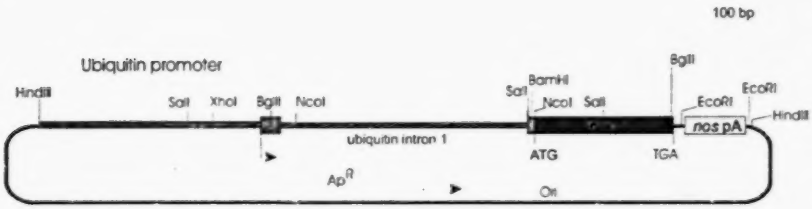


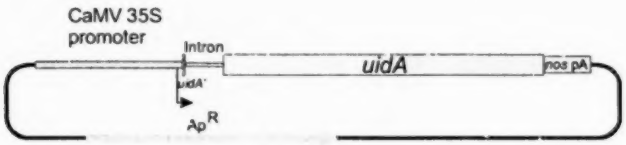
Fig. 6. Schematic illustration of selection vector pUB16.

provides resistance against Geneticin and Paramomycin, which we have used as selection agents in regeneration media for transformed wheat and barley cells and tissues. The maize ubiquitin promoter, expressed at high levels in rapidly growing cells (Cornejo et al. 1993), was used for assembly of the selection vector pUB16 (Fig. 6). The selectable marker gene, *bar*, located on pUB16 encodes resistance to L-PPT, the active ingredient of herbicides Basta and Liberty Link. Both pKA8 and pUB16 carry the nopaline synthase (*nos*) polyadenylation sequence (pA). The pKA8 vector has been used in wheat and barley transformation experiments, whereas the pUB16 construct has mainly been used for transformation of barley (see Milestones 5 and 6).

For optimization of transformation protocols it is desired to insert a transgene that encodes a product that can easily be assayed for. One of these scorable vectors we have assembled is construct p35S-G1-1 (Fig. 7a), which carries the CaMV 35S promoter driving expression of *uidA* (GUS). The GUS gene carried by p35S-G1-1 is interrupted by an intron, and thus, expression of GUS activity can only be detected in cells where the construct has been integrated into the genome. Therefore, this construct is useful for monitoring stable transformation of plant cells. The luciferase gene (*luc*) was used to

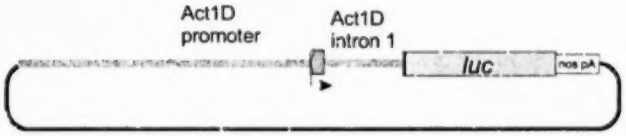
assemble scorable vector pAct-LUC (Fig. 7b), in which the rice actin promoter with its first intron is fused to the coding region of *luc*. Construct pGFP5 (Fig. 7c; Haseloff et al. 1997) carries the CaMV 35S promoter driving expression of a green fluorescent protein (GFP) gene. The GFP activity is localized to the endoplasmic reticulum, as the gene encodes an ER-targeting signal (HDEL) at the end of the coding sequence.

(a)



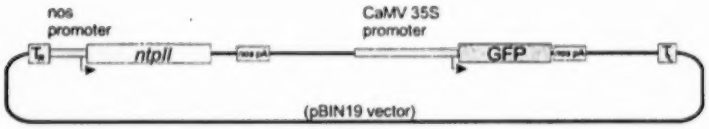
p35S-GI-1

(b)



pAct1D-LUC

(c)



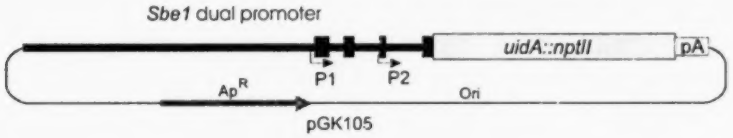
pGFP5

Fig. 7. Monocot expression vectors expressing (a) GUS, (b) luciferase and (c) GFP activities.

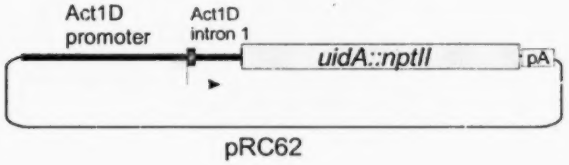
Construct pGK105 (Fig. 8a) represents a hybrid between a selectable and scorable transgene. The gene product, GUS::NPTII, produced by the activity of the dual SBE promoters P1 and P2, gives both β-glucoronidase and neomycin phosphotransferase activity. The GUS::NPTII gene is also carried by construct pRC62 (Fig. 8b) described by Nehra et al. (1994) and used in several of our transformation experiments. On pRC62, the rice actin promoter controls expression of the fusion gene. Construct pBARGUS (Fig. 8c) described by Nehra et al. (1994) was used mainly in transformation experiments with barley. The hybrid protein produced from this construct encodes both GUS activity and

phosphinotricin acetyl transferase (PAT) activity, of which the latter provides protection against the herbicides Basta and Liberty Link.

(a)



(b)



(c)

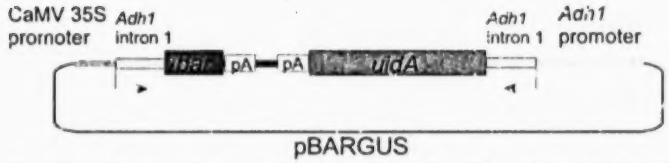


Fig. 8. Schematic outline of expression constructs carrying selectable and scorable marker genes.

Monocot transformation vectors directing expression of anti-sense starch branching enzyme (SBE) transcrips.

A total of twelve vectors with anti-sense starch branching enzyme (*Sbe*) expression cassettes have been assembled. The vectors were constructed by inserting *Sbe* cDNA fragments downstream of the rice actin promoter carried by constructs pAct1a or pAct1c (Figs. 3 and 9a) or the endosperm-specific glutenin promoter (Fig. 10). The cDNA fragment used for these constructions are indicated in Fig. 9 and include sequences from *Sbe2a*, *Sbe2b* and *Sbe1c*, respectively.

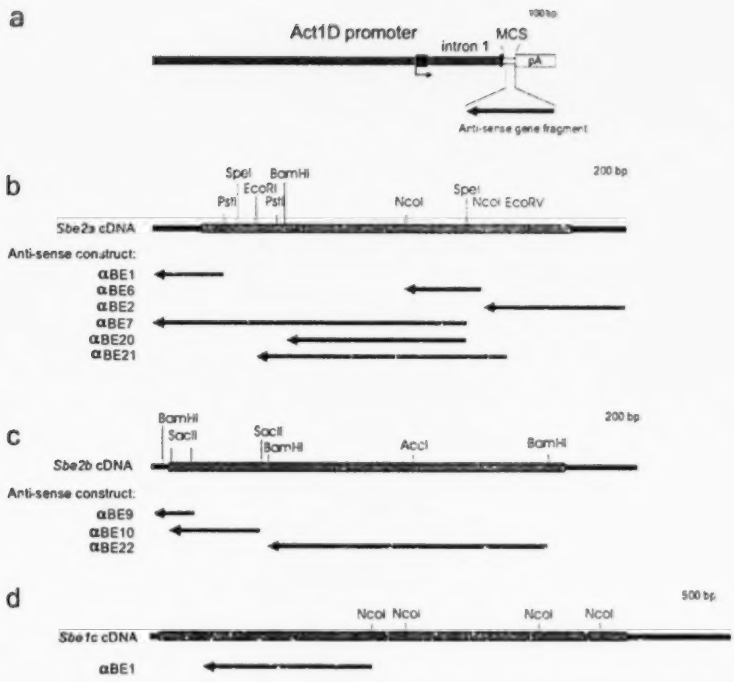


Fig. 9. Schematic illustration of *Sbe* anti-sense expression cassettes controlled by the rice actin promoter. (a) shows expression cassette carried by pAct1a or pAct1c and used for assembly of the anti-sense constructs. Below are illustrated *Sbe2a* (b), *Sbe2b* (c) and *Sbe1c* (c) cDNA clones and fragments thereof (arrows) used for construction of the anti-sense vectors.

Most of the anti-sense vectors presented in Fig. 9 and 10 have been used in wheat transformation experiments to test the function of SBEIc, SBEI and SBEII during starch biosynthesis in wheat endosperm (Milestone 5). Anti-sense expression of *Sbe2* is expected to reduce the amount of branched glucan polymers accumulated in starch granules, thus producing starch with novel properties and uses. Anti-sense expression of *Sbe1* and/or *Sbe1c* may affect granule size distribution.

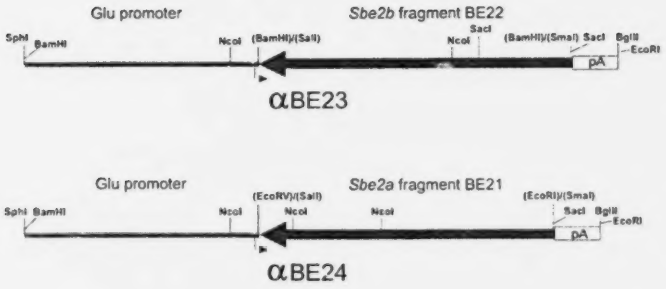


Fig. 10. Schematic illustration of *Sbe2b* and *Sbe2a* anti-sense expression vectors controlled by the endosperm-specific glutenin promoter.

Monocot transformation vector encoding Fusarium resistance.

A plasmid carrying a Fusarium-resistance gene driven by the rice actin promoter (Fig. 11) was obtained from Drs. Linda Harris and Steve Gleddie, Eastern Cereals Oilseeds Research Center, Ottawa. The construct has been used in transformation experiments described in Milestones 5 and 6.

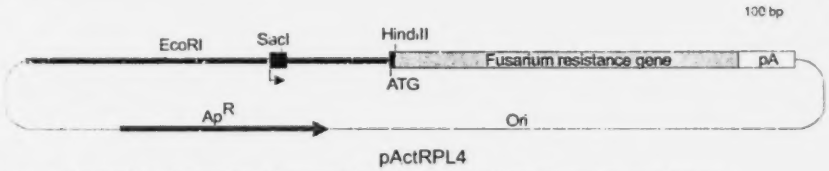


Fig. 11. Schematic illustration of expression vector encoding *Fusarium* resistance.

Construction of hairpin cloning vector.

A cloning vector for assembly of hairpin anti-sense expression constructs (Smith et al. 2000) has been produced. The construct carries the *Sbe1* P1 promoter (Båga et al. patent) including the *Sbe1* first exon, first intron, part of second exon and entire second intron. Multiple cloning sites (MCS) have been inserted immediately upstream and downstream of second intron to allow insertion of gene fragments in sense and anti-sense orientations as illustrated in Fig. 12.

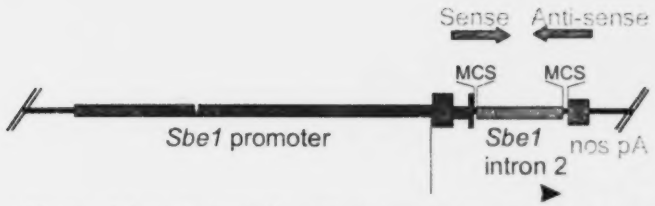


Fig. 12. Schematic illustration of hairpin anti-sense expression cassette.

MAR/SAR sequences.

Genetic transformation of plants generally results in a great variation in expression levels of inserted genes between independent transformants (Peach and Velten 1991). This variability is known as position effects and due to influences of surrounding host DNA on inserted transgene. A reduction in gene variability was reported by several research groups to occur when matrix attachment regions (MAR or SAR) were located on each side of an inserted transgene (e.g. Laemmli et al. 1992). The SAR/MAR sequences were proposed to reduce the position effects by shielding the intervening DNA from influences outside of the SAR/MAR borders. These experiments were mainly conducted in animal systems and provided great expectations that gene silencing could also be reduced in plant systems if inserted expression cassettes were provided with SAR/MAR sequences. Experiments conducted in tobacco supported that transgene variability could be redcued by the presence of SAR/MAR surrounding the transgene (Mlynarova et al. 1996). However, later experiments in rice demonstrated a higher gene expression level but no effect on gene expression variability from constructs carrying SAR/MAR sequences (Vain et al. 1999). Recent experiments conducted in maize have not shown any effect on gene expression levels among independent transformants (Thomas Peterson, Iowa State University, presentation at Plant Animal & Microbe Genomes X meeting, 2002). Thus, there are conflicting results regarding the benefit of SAR/MAR sequences on reduction of transgene expression variability in cereals. Awaiting further support for SAR/MAR sequences, we have eliminated the construction of SAR/MAR containing vectors from our original proposal.

Milestone 2: Comparison of regeneration response of different tissue explants from four wheat genotypes (winter, CPS, HRS and durum)

Immature embryos or scutella isolated from developing kernels have so far been the primary source of explants used for regeneration and transformation of wheat. The age of the kernels at harvest has been found to be important for obtaining optimal regeneration from both types of explants. Development of somatic embryos from isolated explants is induced through cultivation of the explants on sugar-containing MS media supplemented with auxin. For some cultivars, incubation of explants first on an auxin-rich medium followed by an incubation on a medium with lower auxin levels or containing cytokinin has been found to be the optimal regime to induce somatic embryos. Thus, for regeneration of plants, the exact media formulation and the regeneration regime have to be carefully optimized for each wheat cultivar.

We have previously reported development of a transformation protocol for scutella of the soft white spring wheat, Fielder (Nehra et al. 1994). Later, the same protocol was applied for regeneration of durum (cv. Plenty) wheat from scutella explants (Maes et al. 1996). However, immature embryos or scutella of other wheat classes have responded poorly using the regeneration strategy optimized for soft white spring wheat. Especially induction of somatic embryos from scutella isolated from hard red spring wheat cultivars has been difficult.

Regeneration of winter wheat from scutella.

No transformation experiments have been reported for Canadian winter wheat cultivars, although this class often produces high yields. The main reason for lack of regeneration protocols for winter wheat, may be that these lines require a six to eight week cold period (4°C) to induce flowering. Thus, the production of explant tissue (immature embryos or scutella) becomes time consuming and expensive for winter wheat.

To test the tissue culture response for winter wheat, an initial regeneration experiment was set up using scutella of the cultivar Osprey. Explants were isolated as described by (Nehra et al. 1994) and incubated on six different culture media to test the callus-induction potential (Table 1). The media differed in their NO_3/NH_4^+ ratios, type of

sugar (sucrose or maltose) and type of auxin (2,4-D or Picloram) added. All of these factors have been shown to play important roles in determining the quality of embryogenic callus in cereals (Maheshwari et al. 1995). Medium 1, a modified form of the MS^{++} medium used for regeneration of soft wheat lines (Nehra et al. 1994) was used as our control medium.

The experiments showed that the type of sugar added to the media had a strong influence on the quality of callus formed. Sucrose supported a faster callus growth than maltose, but the callus formed on sucrose media had a consistently lower regeneration potential than callus produced on maltose media, as measured by the number of green spots produced. The highest regeneration was obtained on media containing a high ratio between NO_3^- and NH_4^+. Media containing the auxin 2,4-D produced a larger number of plantlets than media containing Picloram. The best regeneration response was obtained with medium 2, containing modified MS^{++} with maltose, 2,4-D and a high NO_3/NH_4^+ ratio. Thus, the optimal regeneration conditions for scutella isolated from the winter wheat cultivar Osprey differed from those of the soft white wheat cultivar Fielder. However, the regeneration frequencies recorded for Osprey were three to five times lower than those obtained with soft white spring wheat cultivars. Small regeneration experiments were also conducted with scutella of winter wheat cultivars, Harrier and Clair, showing similar regeneration responses as found for Osprey.

Table 1. Regeneration experiment with Osprey, a winter wheat cultivar.

Media	# scutella	NO_3^-/NH_4^+ ratio	Sugar	Auxin	Callus induction frequency (%)	Calli with green spots (%)	Plants per callus
1	300	High	Sucrose	2,4-D	92	19	3
2	300	High	Maltose	2,4-D	99	51	4
3	300	High	Sucrose	Picloram	89	30	0
4	300	High	Maltose	Picloram	88	35	0
5	175	Low	Sucrose	Picloram	76	33	0
6	280	Low	Maltose	Picloram	95	2	0

One of the major problems encountered with regeneration of winter wheat from scutella, was the high number of explants producing roots instead of shoots. To reduce the rooting problem, $AgNO_3$ was added to the media to test if the ethylene inhibitor would reduce rooting as previously reported (Purnhauser et al. 1987). However, presence

of $AgNO_3$ did not improve the regeneration frequencies for winter wheat. We also tested if lower levels of 2,4-D in the media would reduce rhizogenesis, but no such effect was found. In summary, scutella and immature embryos were found to be poor explants for regeneration of the winter wheat cultivars used in this study.

Regeneration of winter wheat from leaf bases.

Isolated leaf base segments containing the primary meristem have been reported to induce shoot development in both barley (Becher et al. 1992) and oat (Gless et al. 1998). In this project we explored the possibility to use isolated leaf bases as explants for three Canadian winter wheat cultivars; Osprey, Harrier and Clair. The main advantage of leaf-bases over scutella is that the leaf bases are isolated from germinating seeds. Thus, there is no need to grow donor plants, and thus, the time needed to produce explant tissue can be shortened by about 16 weeks.

Germinating seeds were produced by sterilization of mature seeds followed by incubation on wet filter papers as described (Gless et al. 1998). After a few days, the germinating shoots were excised and incubated onto different media. Our first experiments were performed with callus initiation media 2 and 6 (Table 1), which stimulated a very high callus production (>95%) for all three cultivars tested. However, subsequent incubation revealed that the embryogenic potential of the callus was poor. Only leaf bases containing the main meristem produced green spots that subsequently regenerated into shoots. Medium 2 induced a higher number of shoots than medium 6, and on average, three shoots per explant were formed.

Direct shoot formation, without production of an intermediate callus (Fig. 13), was demonstrated when leaf bases were incubated on a modified Medium 2 containing an unclassified growth regulator (UGR). About 50% of the isolated Osprey leaf bases produced shoots, and on average, two to three shoots were formed per leaf base. Direct shoot induction was an important improvement of the leaf base regeneration protocol, since it reduced the time for production of mature plants.

Fig. 13. Formation of multiple shoots, without intermediate callus formation, from leaf base of the winter wheat cultivar Osprey.

Regeneration of winter wheat from mature embryo axes.

We also tested if mature embryo axes of winter wheat, like leaf bases, could be induced to produce multiple shoots. In an experiment with the winter wheat cultivars Osprey and Clair, it was found that about 90% of the isolated mature embryo axes produced multiple shoots and on average, three shoots were formed per axis. As compared to leaf bases, mature embryo axes as explant material had a similar or higher shoot regeneration rate. Furthermore, the mature embryo were much easier to excise and cultivate than leaf bases and could be processed in a shorter time than leaf bases, since seed germination was not needed.

We also compared multiple shoot induction frequencies for mature and immature embryos axes. These regeneration studies were combined with transformation by particle bombardment. The results showed that mature embryo axes regenerated equally well or better than immature embryo axes (Table 2). Thus, mature embryo axes, which are easier to obtain in large numbers than immature axes or scutella, were used as explant material in a few transformation experiments with winter wheat.

Table 2. Regeneration frequencies obtained from mature and immature embryo axes of winter wheat.

Cultivar	Explant type	Total # axes	# axes with shoots	% regeneration
Osprey	Immature embryo axes	226	160	70.8
Osprey	Immature embryo axes	355	321	90.4
Osprey	Immature embryo axes	82	77	93.9
Osprey	Mature embryo axes	316	300	94.9
Osprey	Mature embryo axes	376	349	92.8
Clair	Immature embryo axes	192	178	92.7
Clair	Immature embryo axes	272	241	88.6

Regeneration of Canadian Prairie Spring (CPS) wheat from inflorescence tissue.

Regeneration of plants from segments of isolated wheat inflorescences was first demonstrated by Ozias-Akins and Vasil (1982). Later studies with *Tritordeum* inflorescences have shown that the length (age) of the explant, incubation media and regeneration strategy are important factors for optimal tissue culture (Barcelo et al. 1989; Rasco-Gaunt and Barcelo, 1999).

In our regeneration experiments using wheat inflorescences, the donor plants were grown under optimal conditions in a greenhouse or growth cabinet. Before the head emerged, stem section containing the inflorescence was cut from the plants and surface-sterilized. Three to 15 mm long inflorescences were excised from stems using a dissecting microscope. The explants were further cut into 1-2 mm sections, which were incubated on an initiation media with 50 mg/L Cefotaxime. In initial experiments performed with the CPS cultivar AC Karma, we tested two different callus initiation media, medium A (Barcelo et al. 1994) and a modified version of medium A (Table 3). The two media differed in sugar concentration and presence/absence of an anti-oxidative agent. Both media were found to support development of a highly embryogenic callus from bombarded inflorescences.

After three to four weeks of incubation on initiation media, explants were transferred to regeneration media containing the appropriate selection agent. Thereafter, the explants were moved to fresh media with selective agent every 2-3 weeks. After two to three round of selection, surviving shoots were transferred to jars with 0MS media for rooting. Rooted shoots were planted in soil and grown in a greenhouse. A comparison of the regeneration frequencies using medium A and it's modified version showed three-fold higher number of shoots from explants incubated on the modified medium (Table 3). Thus, we chose to use the modified medium A for the callus initiation in subsequent regeneration and transformation studies with inflorescences of wheat cultivars.

Table 3. Evaluation of two callus induction media for regeneration of Paramomycin-resistant CPS wheat from bombarded inflorescences.

Media	Inflorescence length (mm)	# explant segments		# explant segments with green spots		# shoots surviving after 2 weeks selection		# shoots surviving after 4 weeks selection		# shoots per segment (%)	
		Exp.	Control	Exp.	Control	Exp.	Control	Exp.	Control	Exp.	Control
A	<10	166		160		20		9		5.4	
	10-15	122	30	112	29	15	3	3	0	2.5	0
	15-20	55		49		6		2		3.6	
A modified	<10	55		50		24		5		9.1	
	10-15	68	20	66	19	21	1	15	1	22.0	5.0
	15-20	70		68		17		4		5.7	

Regeneration of hard red wheat from inflorescences.

Hard red wheat is the most difficult class of Canadian wheat to regenerate using scutella as explant material. At best, only a few shoots are formed per explant, which is too low for transformation experiments. To find a more suitable explant tissue for hard red wheat, we have explored the possibility to use inflorescences. The initial experiments were performed with wheat line BW720 and conducted as described above for CPS AC Karma. These experiments showed a much better regeneration response from inflorescences than scutella. However, as compared to AC Karma, the regeneration frequencies for the hard red cultivar were three to six times lower. A few transformation experiments with a hard red wheat line have also been done (see Milestone 5).

Milestone 3: Comparison of regeneration response of different tissue explants from four barley genotypes (malting, feed hulless and hulled).

For genetic transformation of barley, the most commonly used explants have been isolated immature embryos or scutella, immature anthers, microspores and leaf-bases. These explants have been preferred partly because of a reasonably good regeneration frequency and partly because of adequate success in genetic transformation experiments. However, all these explant tissues are not readily available. Donor plants have to be grown under highly controlled conditions for several weeks-months, before explant tissues can be harvested. The time-span for obtaining the explants varies from 3-10 days for isolation of leaf-bases to six weeks for isolation of scutella. It would be more cost-effective if donor materials did not have to be grown in growth chambers or greenhouses. Although leaf-bases offer such a possibility, their isolation still remains labor-intensive,

but still simpler and less labor-intensive than the other explants mentioned. We have successfully completed Milestone 3 by setting up a number of experiments aimed at comparing regeneration frequencies from different explants (immature scutella, leaf-bases and mature embryos) of four barley genotypes, namely, CDC Dawn, CDC McGwire (feed barleys), CDC Copeland (2-row malting barley) and BT459 (6-row malting barley). Different media compositions were tested using explants from these four genotypes.

Use of immature scutella as explant tissue for regeneration of barley.

The four barley genotypes (CDC Dawn, CDC McGwire, CDC Copeland and BT459) used for optimization of regeneration were grown in growth chambers under controlled conditions. Spikes were harvested 10-12 days post-anthesis and used either immediately or stored at 4°C for up to three days. For isolation of immature scutella, caryopses were de-huiled, surface-sterilized in 5% commercial bleach for five minutes and rinsed several times in sterile deionized water. Scutella were isolated using a dissection microscope and transferred to callus induction media.

To induce embryogenic callus, media Mbar and MB1 were tested. MBar has previously been shown to be efficient with other barley cultivars (Weir et al., 1998). It contains basal MS medium (Murashige and Skoog, 1962), Gamborg's B5 vitamins (Gamborg et al., 1968), 3% maltose and 1 mg/L 2,4-D. MB1 has a similar composition as Mbar, with the exception that 1 g/L casein hydrolysate, 0.7 g/L L-proline and 5 μM $CuSO_4$ were added. Medium MB2 is as MB1, but with higher 2,4-D (2.0 mg/L) concentration. Table 4 shows the regeneration response from immature scutella-derived calli.

Table 4. Callus induction frequencies from immature scutella of barley.

	Callus induction frequency (# calli / # explants)		
Genotype	Mbar medium	MB1 medium	MB2 medium
BT459	300/360 (83%)	567/583 (97%)	395/395 (100%)
Dawn	334/600 (56%)	561/600 (94%)	458/510 (90%)
McGwire	334/600 (56%)	564/600 (94%)	ND
Dawn	-56%	534/600 (89%)	-86%
Copeland	ND	-99%	ND

ND = no experiment conducted.

Our experiments indicated that scutella of all four genotypes responded favorably on medium MB1 and produced highly embryogenic calli (Fig. 14). The callus induction frequency was as high as 99%. Although on medium MB2, the callus induction frequency appeared to be higher, the calli produced was brownish in color; an indication of low embryogenic potential. The callus induction frequency on medium MBar was very low compared to media MB1 and MB2. Although compact, nodular and yellowish embryogenic calli were obtained, the low frequency of such calli

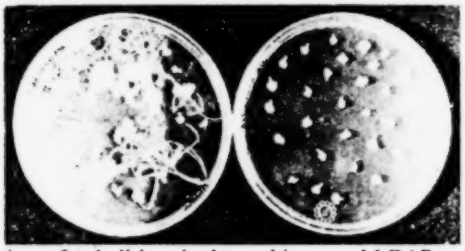


Fig. 14. Regeneration of a hull-less barley cultivar on M-BAR medium supplemented with amino acids. Culture dish to the left contains a higher level of amino acids than dish to the right.

would not translate into an efficient regeneration frequency. Based on these observations, we decided to conduct all subsequent experiments for callus induction on medium MB1.

Calli induced on MB1 from immature scutella of all four genotypes of barley were subsequently tested for their regeneration efficiency on six different media (Table 5). Previous studies with other genotypes of barley (Weir et al., 1998) have shown that Mbar was also effective for regeneration, besides callus induction. Therefore we tested the culture media Mbar and MB1 for regeneration efficiency. Four other media containing increasing levels of the cytokinin, BAP, and low levels of 2,4-D were also tested. FHG, a medium reported to be efficient for regeneration from barley microspores (Hunter, 1988) was also tested, as it was reported to be effective for regeneration of plants from immature barley embryos (Cho et al. 1999). However, we were not successful in reproducing the results obtained by Cho et al. (1999) with our barley genotypes. All the calli on FHG medium turned brown within three weeks and died.

Table 5. Shoot regeneration frequency from scutella-derived callus cultivated on different media.						
	Regeneration frequency (regenerated shoots per explant) on medium:					
Genotype:	Mbar	MB1	MBR1	MBR2	MBR3	MBR4
Dawn	17/150 (0.11)	79/150 (0.53)	76/150 (0.51)	18/90 (0.2)	533/360 (1.5)	613/240 (2.6)
McGwire	56/150 (0.37)	58/60 (0.97)	87/150 (0.58)	29/60 (0.48)	339/150 (2.3)	424/150 (2.8)
Copeland	53/120 (0.44)	69/90 (0.77)	123/240 (0.51)	76/90 (0.84)	213/120 (1.8)	468/210 (2.2)
BT459	96/240 (0.4)	84/150 (0.56)	78/180 (0.43)	68/90 (0.76)	197/120 (1.6)	226/150 (1.5)

With the media containing increasing levels of BAP (MBR1, MBR2, MBR3 and MBR4), MBR3 and MBR4 provided better regeneration than media MBR1 and MBR2. Regeneration of plants on medium MBR4 was slightly better than on medium MBR3. However, the shoots on MBR4 were more slender, which made separation for rooting difficult due to appreciable damage. Thus, regeneration in subsequent experiments was mostly carried on medium MBR3.

Use of leaf-base segments as explant tissue for regeneration of barley.

As opposed to immature scutella, leaf-bases can be obtained fairly easily and rapidly, and are less labor-intensive for isolation. Leaf bases, as a source of explant tissue for genetic transformation, has been demonstrated for oat (Gless et al., 1998) and barley (Zhang et al., 1998), albeit in barley through direct multiple shoots. We set forth, in this part of the investigation, to initiate callus from leaf-bases and to subsequently regenerate plants, as well as to attempt induction of direct multiple shoots from leaf-bases. Mature seeds were surface-sterilized, germinated in the dark for three days on agar solidified MS medium and 1-2 mm leaf-bases were excised for callus induction. The four barley cultivars mentioned above were again tested. For callus induction, the excised leaf-bases were placed on medium MB1, as this medium was shown to produce good embryogenic calli with immature scutella. The calli obtained were transferred for regeneration onto MB1 and the four other regeneration media (MBR1, MBR2, MBR3 and MBR4). Table 6 shows the regeneration frequencies obtained from leaf-base calli from the four genotypes on the different regeneration media. Medium MBR4 appeared to be most suitable for genotypes CDC Dawn, CDC McGwire and BT459, while medium MBR3 was more suitable for CDC Copeland.

Table 6. Shoot induction frequencies from leaf base-derived callus incubated on different media.					
	Regeneration frequency (# regenerated shoots per explant) on medium:				
Genotype:	MB1	MBR1	MBR2	MBR3	MBR4
Dawn	57/78 (0.73)	107/104 (1.02)	86/78 (1.1)	87/104 (0.84)	271/182 (1.5)
McGwire	18/52 (0.35)	69/78 (0.88)	93/104 (0.89)	94/104 (0.9)	126/104 (1.7)
Copeland	140/130 (1.1)	132/104 (1.3)	81/78 (1.04)	239/74 (3.2)	184/78 (2.4)
BT459	82/104 (0.79)	87/104 (0.84)	88/104 (0.85)	114/104 (1.1)	91/78 (1.2)

Use of mature embryo as explant tissue for regeneration of barley.

Mature embryos present an appealing explant system, as they are readily available and do not require growth of donor plants. We attempted to use mature embryos for regeneration of barley and optimize regeneration from such explants. Dry seeds were surface-sterilized in commercial bleach for 45 min, rinsed several times in sterile water and imbibed overnight at 4°C. Mature embryos were isolated using a dissection microscope and transferred to medium MB1 for callus induction. However, the callus induced was watery and not very embryogenic. Upon further subculture, the calli did turn embryogenic but plant regeneration frequencies were very low (Table 7). No further experiments were carried out using mature embryos for callus induction and regeneration. Our results are in agreement with those of Lupotto (1984), wherein low frequency of regeneration was also observed.

In an attempt to mimic the leaf-base culture system of Zhang et al. (1998) to produce direct multiple shoots, we placed the mature embryo explants directly on our regeneration media containing different levels of BAP (MBR1, MBR3 and MBR4) and on medium MB1. On the latter medium, as mentioned above, callus induction occurred and upon two sub-cultures some embryogenic callus was produced. Regeneration frequency on medium MB1 was less than one shoot per explant (Table 7). Media MBR1, MBR3 and MBR4 were all able to induce direct multiple shoots at different efficiencies. Medium MBR1 was the least effective, producing only one shoot per explant. On medium MBR3, only CDC Copeland produced about two shoots per explant. Medium MBR4 appeared to be the most effective for induction of multiple shoots from all four barley genotypes, in the range of 2-4 shoots per explant.

Table 7. Shoot induction frequencies from barley mature embryos on different media.

	Medium			
	MB1	MBR1	MBR3	MBR4
Genotype				
Dawn	69/78 (0.88)	107/104 (1.0)	183/156 (1.2)	271/78 (3.5)
McGwire	98/104 (0.94)	169/156 (1.1)	94/78 (1.2)	229/78 (2.9)
Copeland	140/156 (0.9)	132/130 (1.0)	239/104 (2.3)	184/78 (2.4)
BT459	82/104 (0.79)	87/78 (1.1)	114/78 (1.5)	191/104 (1.8)

Improved direct multiple shoot induction from leaf-base and mature embryo explants.

Encouraged by the results obtained using mature embryo explants on BAP containing media for direct multiple shoot induction from barley, we decided to try the cytokinin zeatin riboside with mature embryo explants from CDC McGwire and CDC Copeland to test if there is an improvement in shoot induction frequency. An unclassified plant growth regulator (UGR) with cytokinin-like properties was also tested.

Table 8. Multiple shoot induction from mature embryos of barley on media containing different levels of zeatin riboside.

	Regeneration frequency (# shoots regenerated per explant) on medium:		
Genotype:	**MBZ1**	**MBZ2**	**MBZ3**
McGwire	98/40 (2.4)	203/180 (2.0)	155/80 (3.4)
Copeland	118/58 (1.8)	136/52 (2.6)	244/110 (3.5)

Table 8 shows the results obtained on media containing zeatin riboside. Although this study may be considered small with respect to the number of explants inoculated, there is a good indication that even zeatin riboside is capable of inducing direct multiple shoots from mature embryos. Medium MBZ3, which contains a higher level of zeatin riboside, appears to be the best medium for induction of multiple shoots.

The unclassified growth regulator (UGR) showed an even higher regeneration frequency for all genotypes tested than media containing BAP or zeatin riboside (Table 9). Media MB5 and MB6 contained 1 mg/L 2,4-D and two different levels of UGR, whereas MBT1 and MBT2 contained solely the two levels of UGR. Medium MB5 induced production of 4-7 shoots per explant, with CDC McGwire showing about 7

shoots. On medium MB6, the number of shoots per explant ranged from 3-8, again with CDC McGwire showing the highest number.

Table 9. Shoot regeneration frequencies from mature embryo explants of barley on different media containing an unclassified growth regulator.

	Regeneration frequency (# shoots regenerated per explant) on medium:			
Genotype:	**MB5**	**MB6**	**MBT1**	**MBT2**
Dawn	347/56 (6.2)	463/104 (4.5)	606/63 (9.6)	330/56 (6.4)
McGwire	516/78 (6.6)	627/78 (8.0)	462 / 61 (7.6)	353 / 48 (7.4)
Copeland	427/78 (5.5)	228/78 (2.9)	2733/216 (8.0)	1132/182 (6.2)
BT459	386/104 (3.7)	394/104 (3.8)	2457/182 (13.5)	1241/182 (6.8)

When the media were supplemented with the UGR alone (MBT1 and MBT2), an even greater number of shoots were produced. On medium MBT1, shoot production ranged from 8-14 shoots per explant, with BT459 displaying the highest number. On medium MBT2, the effect was less pronounced than medium MBT1, in the range of 6-7 shoots per explant. The total number of shoots per mature embryo could be increased further by prolonged incubation on the induction medium. Thus, it appears that the UGR as a sole growth regulator in the medium is more potent for induction of multiple shoots from mature embryos of barley.

In another small experiment, the combinations of the UGR and BAP were evaluated to see if the UGR and BAP interact in a synergistic manner to further enhance the frequency of shoot induction. Two levels of the UGR, as used above in media MBT1 and MBT2, and two levels of BAP (1 and 2 mg/L) were used in permutation and combination to make up media T1B1, T1B2, T2B1 and T2B2. Only some genotypes were tested and the results are shown in Table 10. However, no definite trend could be deduced. BT459 showed the highest response (10.5 shoots per explant) on medium T1B2, which contained a low level of UGR and a high level of BAP. When UGR was used alone (Table 10), 13.5 shoots per explant were produced. High level of UGR in combination with low level of BAP showed an increase in shoot production from BT459 as compared to MBT2 alone. High level of both growth regulators showed a decrease in BT459 shoot production compared to UGR used alone. It may be possible that BAP is

not acting synergistically and probably interferes with the mode of UGR action.

Table 10. Shoot regeneration frequencies from mature embryo explants of barley on different media containing an unclassified growth regulator and BAP.

	Regeneration frequency (# shoots regenerated per explant) on medium:			
Genotype:	**T1B1**	**T1B2**	**T2B1**	**T2B2**
Dawn	167/41 (4.1)	148/39 (3.8)	ND	ND
McGwire	ND	ND	ND	ND
Copeland	727/84 (8.7)	571/80 (7.1)	405/55 (7.4)	514/86 (6.0)
BT459	301/35 (8.6)	169/16 (10.5)	491/58 (8.5)	291/59 (4.9)
ND = Not done				

Further detailed studies need to be carried out to elucidate this interaction. Increasing the levels of the UGR to levels greater than those present in MBT1 and MBT2 was not beneficial (Fig. 16). The explants turned brown, exhibiting a scorched appearance within a few days. This indicates that the UGR has a certain optimal level for its activity as a growth regulator, and beyond that it becomes phytotoxic.

Fig. 15. Regeneration of a hull-less barley cultivar from leaf-base cultures incubated on Mbar medium with different levels of an unclassified plant growth regulator added. The culture dishes to the left and right, respectively, contain different levels of the compound.

Leaf-base explants were also tested on the two media containing the UGR and 2,4-D (MB5 and MB6) in comparison with two media containing high levels of BAP (MBR5; 3 mg/L and MBR6; 4 mg/L). Table 11 shows the result of this study. Multiple shoot production did occur on MB5 and MB6, but not on MBR5 and MBR6. On MB5, the shoot regeneration was in the range of 2-8 shoots per explant, with CDC McGwire showing the highest frequency. On MB6, again CDC McGwire showed the best response. Use of the UGR alone was not tested with the leaf-bases, since the simplicity and efficiency of mature embryos were demonstrated earlier (Table 9). MBR5 and

MBR6 were tested to see if higher levels of BAP could produce the same responses as the UGR.

Table 11. Multiple shoot induction frequencies from barley leaf-bases using different media.

	Regeneration frequency (# shoots regenerated per explant) on medium:			
Genotype:	**MB5**	**MB6**	**MBR5**	**MBR6**
Dawn	247/52 (4.8)	263/52 (5.1)	115/120 (0.96)	97/104 (0.93)
McGwire	217/26 (8.3)	367/56 (6.6)	178/208 (0.86)	162/156 (1.03)
Copeland	227/104 (2.2)	328/130 (2.5)	76/182 (0.42)	54/104 (0.52)
BT459	186/78 (2.4)	191/78 (2.4)	145/104 (1.4)	103/104 (1.0)

Milestone 4: Construction of super-binary vectors containing expression cassettes in Milestone 1 and engineering of *Agrobacterium* strains to harbor super-binary vectors.

Assembly of Agrobacterium co-integrate vectors.

Fig. 16 illustrates two *Agrobacterium* co-integrate vectors that have been assembled using cloning vectors provided by the Japan Tobacco System, under a licence agreement. The two constructs contain the *nptII* and *bar* expression cassettes, respectively, isolated from plasmids pKA8 and pUB16 presented in Milestone 1. *Agrobacterium* strains carrying the pSBIII-KA or the pSBIII-UB vector have been used in transformation of wheat and barley, respectively.

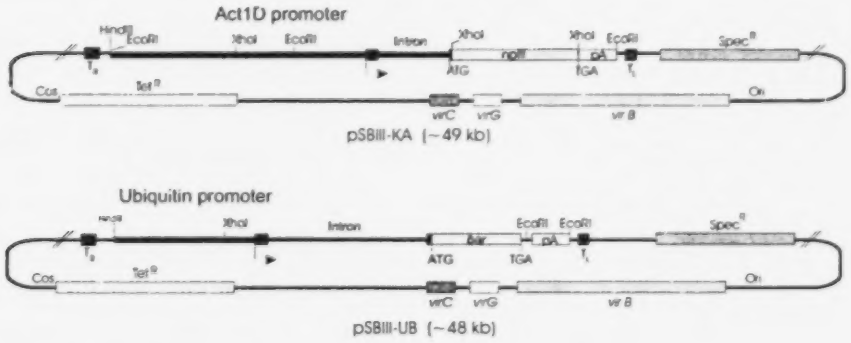


Fig. 16. Schematic organization of two co-integrate vectors carrying T-DNA cassettes with selectable marker genes.

Construction of Agrobacterium plasmid system with two T-DNA cassettes.

The use of *Agrobacterium* strains carrying two separate T-DNA cassettes, one carrying expression unit for the selectable marker and the other expression unit for the gene of interest, would be of great value for production of transgenic wheat and barley. The main advantage lies in the independent insertion of the two cassettes into the plant genome. This will allow separation of the marker gene and gene of interest with a high frequency in the following generations (Komari et al. 1998), leading to production of transgenic plants devoid of antibiotic- (or herbicide-) resistance genes. To use this system, we have modified the *Agrobacterium* transformation vector pSB1 (Japan Tobacco) to include a T-DNA cassette carrying a selectable marker gene (Fig. 17).

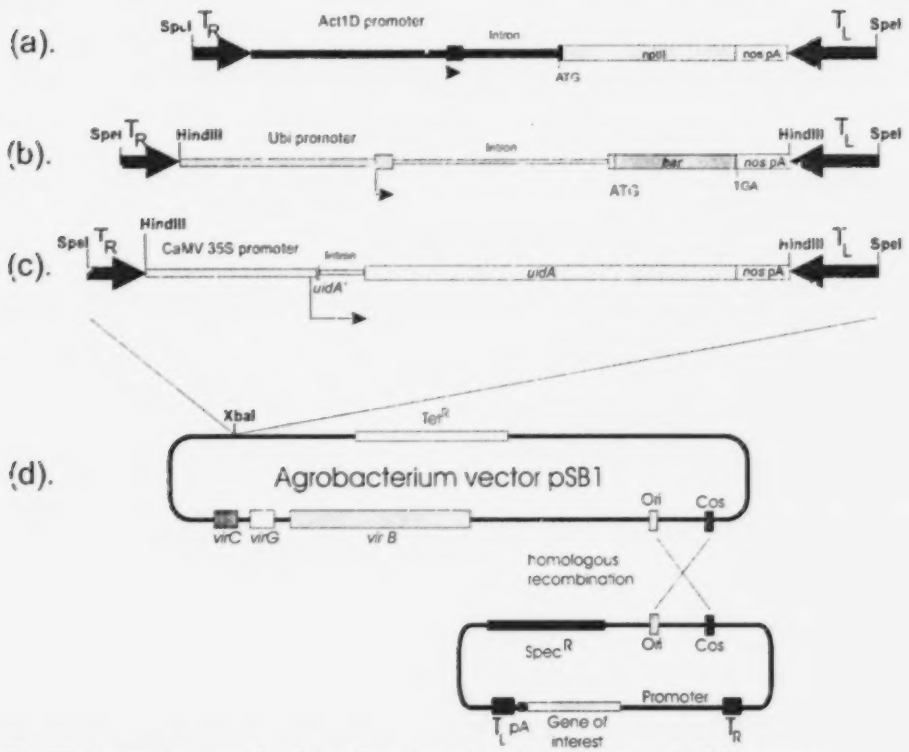


Fig. 17 Strategy for assembly of *Agrobacterium* vector with two separate T-DNA cassettes.

For these constructions, the following expression cassettes with selectable marker genes were used: (a) the actin promoter driving *nptII* expression isolated from construct pKA8, (b) the ubiquitin promoter driving the *bar* expression isolated from pUB16 and (c) the CaMV 35S promoter controlling a *uidA*-intron-*uidA* isolated from construct p35S-G1-1. Insertion of a second T-DNA cassette with gene of interest into the modified pSB1 construct can be done by conjugation and homologous recombination as outlined in (d).

Several reports have indicated that the choice of *Agrobacterium* strain may be of importance for the overall success of gene delivery and insertion into the plant genome. So far, all of our experiments using *Agrobacterium* transformation experiments have been done with strain LBA4404. To test if other strains are better suited for gene delivery into wheat and barley, we have obtained additional *Agrobacterium* strains (Table 12). Three of these strains have been transformed with plasmid pTOK233.

Table 12. *Agrobacterium* strains used in transformation experiments of wheat and barley.

Strain	Characteristics
LBA4404	octopine
EHA101	agropine/succinamopine; supervirulent
EHA10 5	agropine/succinamopine
GV3111	octopine type
MP90RK	nopaline type

Milestone 5: Genetic transformation of selected wheat genotypes with biolistics and Agrobacterium mediated gene delivery.

Results from the regeneration experiments described in Milestone 2 were used to determine the most suitable explant tissue to be used for transformation of cultivars belonging to the different classes of wheat. The constructs used in the transformation experiments are all described in Milestones 1 and 4. The transgenes introduced include selectable marker genes, scorable marker genes such as *uidA* (GUS), various anti-sense

constructs for starch branching enzyme (αSBE1 αBEII constructs) and a construct carrying a Fusarium-resistance gene (pActRPL4). In total, we have obtained 608 putative transgenic plants belonging to five different classes of wheat (soft white spring wheat, CPS white, durum wheat, red winter wheat and hard red spring wheat (Tables 13, 14, 15).

Transformation of wheat scutella using biolistics.

Our previous regeneration experiments have shown that isolated scutella of soft white spring wheat and durum wheat respond well in tissue culture (Nehra et al. 1994). This explant tissue was therefore chosen for most transformation experiments with cultivars Fielder and Nanda (soft white spring) and Plenty (durum). The scutella were isolated from surface-sterilized seeds that had been collected 12-13 days post anthesis and incubated at 4°C prior to scutella isolation. Excised scutella were incubated for one to three days on MS^{++} medium containing cefotaxime (100 mg/L) to suppress any bacterial contamination. Gold particles (0.6 µm; Bio-Rad) were coated with DNA constructs as described by Nehra et al. (1994) and delivered to the target tissues using a Bio-Rad PDS 1000/He gun. In most experiments, the gun pressure was set 1100 psi, vacuum at 25 inch Hg, and a gap distance of 2.5 cm was chosen. The distance to the target tissue was 7.5 cm and a stopping plate with a 0.8 cm aperture was used in all experiments. After bombardment, the explants were incubated in the dark at 25°C on cefotaxime-containing MS^{++} medium for two days and thereafter placed on plain MS^{++} medium for 10-14 days. To select for shoots transformed with selectable marker gene, the explants were transferred to regeneration media containing the selective agent, Geneticin, Paramomycin or L-PPT (phosphinothricin) at concentration optimal for each cultivar. The selection step lasted for five to seven weeks with transfer to fresh media every second week until non-bombarded explants did not show any green shoots. Surviving shoots derived from bombarded explants were transferred to half-strength MS medium for rooting and thereafter planted in soil. Plantlets of transformed winter wheat cultivars were subjected to a six week cold-treatment to induce flowering.

Transformation experiments with the soft white spring wheat cultivars, which is the easiest wheat class to transform, produced 340 putative transgenics (Table 13). Five Clair (winter) and six Plenty (durum) putatively transgenic lines were also obtained from these experiments with scutella.

Table 13. Regenerated plants from bombarded scutella.

Class: cultivar	Experiment	Selectable + scorable marker plasmids	# regenerated plants
Soft white spring wheat:			
Fielder	AF11	pKA8 + aBE7	11
	AF22	pKA8 + aBE21	1
	AF23	pKA8 + aBE21	2
	AF68	pKA8 + aBE23	5
	AF69	pKA8 + aBE23	14
	AF70	pKA8 + aSBEI	24
	AF71	pKA8 + aSBEI	8
	AF605	pKA8 + aBE24	3
	AF606	pKA8 + aSBEI	2
	AF607	pRC62 + pActRPL4	19
	AF608	pRC62 + pActRPL4	1
	AF609	pKA8 + aBE24	4
	AF611	pRC62 + pActRPL4	16
	AF613	pKA8 + aBE23	15
	AF614	pKA8 + aBE23	2
	AF615	pRC62 + pActRPL4	2
	AF616	pKA8 + aBE23	2
	AF617	pKA8 + aBE23	20
	AF618	pRC62 + pActRPL4	1
	AF619	pKA8 + aBE23	4
			Total = 167
Nanda	AF76	pKA8 + aBE23	3
	AF77	pKA8 + pActRPL4	9
	AF78	pKA8 + pActRPL4	4
	AF79	pKA8 + aSBEI	6
	AF80	pKA8 + aSBEI	2
	AF81	pKA8 + aSBEI	1
	AF82	pKA8 + aSBEI	2
	AF100	pKA8 + pActRPL4	1
	AF102	pKA8 + pActRPL4	7
	AF103	pKA8 + pActRPL4	1
	AF104	pKA8 + pActRPL4	1
	AF105	pKA8 + aSBEI +aBE23	4
	AF106	pKA8 + aSBEI +aBE23	2
	AF109	pKA8 + aBE23	1
	AF110	pKA8 + aSBEI	1
	AF112	pKA8 + aBE23	10
	AF401	pKA8 + pActRPL4	12
	AF402	pKA8 + pActRPL4	8
	AF403	pKA8 + pActRPL4	88
	AF404	pKA8 + pActRPL4	10
			Total = 173
Winter wheat:			
Clair	AF135	pUB16 + aBE24	5
			Total = 5
Durum wheat:			
Plenty	P37	pBARGUS + pActRPL4	4
	P39	pBARGUS + pActRPL4	1
	P40	pBARGUS + pActRPL4	1
			Total = 6

Transformation of wheat immature embryos using biolistics.

Particle bombardment and selection of transformed immature embryos was performed as described for isolated scutella. This experiment was only done with winter wheat and produced 119 regenerated plants from four experiments. However, preliminary screening of regenerated plants indicates that most of plants surviving selection were escapes.

Table 14. Regenerated plants from bombarded immature embryos.

Winter wheat	Experiment	Selectable + scorable marker plasmids	Number of regenerated plants
Clair	AF142	pKA8 + aBE23	2
	AF143	pUB16 + aBE23	66
Osprey	AF144	pUB16 + aBE23	3
	AF145	pRC62 + aBE23	48
			Tot. = 119

Transformation of wheat inflorescences using biolistics.

Inflorescences were in our regeneration experiments were found to be a good source of explant material for *in vitro* regeneration of the CPS cultivar Karma. The inflorescences explants used for our transformation experiments were isolated as described in Milestone 2 and bombarded as described above for scutella. The bombarded tissues were incubated on the modified medium A for three to four weeks with one transfer to fresh medium after one week. Thereafter, selection for transformed shoots was initiated by transfer to regeneration medium containing the antibiotic Paramomycin (25 mg/L). After two weeks selection at low level, the developing shoots were transferred to regeneration medium with a higher Paramomycin concentration (50-60 mg/L). Shoots surviving the stringent selection for four to six weeks were transferred to half-strength MS medium for rooting. Rooted shoots were planted in soil and grown in a green-house to maturity. In total, 129 CPS and nine hard red spring wheat lines putatively carrying transgenes were recovered from the transformation experiments with inflorescences.

Table 15. Regenerated plants from bombarded inflorescences.			
Class: cultivar	**Experiment**	**Selectable + scorable marker plasmids**	**# regenerated plants**
CPS wheat:			
Karma	AF14	pKA8 + aSBEI	1
	AF16	pKA8 + aBE20	6
	AF17	pKA8 + aBE22	10
	AF18	pKA8 + aBE22	3
	AF19	pKA8 + aBE22	2
	AF25	pKA8 + aBE23	1
	AF39	pKA8 + aBE21	1
	AF46	pKA8 + aBE23	2
	AF47	pKA8 + aBE23	3
	AF48	pKA8 + aBE24	13
	AF49	pKA8 + aBE24	10
	AF64	pKA8 + aBE23	4
	AF65	pKA8 + aBE23	1
	AF66	pKA8 + aBE23	2
	AF67	pKA8 + aBE23	2
	AF72	pKA8 + aSBEI	1
	AF84	pKA8 + aSBEI	2
	AF88	pKA8 + aSBEI	1
	AF89	pKA8 + aSBEI	1
	AF101	pKA8 + pActRPL4	16
	AF178	pUB16 + aBE23	2
	AF179	pKA8 + aBE24	3
	AF180	pKA8 + aBE23	11
	AF181	pKA8 + aBE23	31
			Tot. = 129
Hard red wheat:			
BW720	AF56	pKA8 + aBE24	8
	AF61	pKA8 + aBE23	1

Transformation experiments using Karma inflorescence and Agrobacterium.

Several transformation experiments using isolated inflorescences and the *Agrobacterium tumefaciens* strain LBA4404 carrying the pTOK233 plasmid have been done. Explant tissues were isolated from the CPS cultivar Karma as described in Milestone 2. The pTOK233 plasmid contains a GUS gene with an intron, which allows determination of transgene delivery to the nucleus of infected cells by staining the tissues with X-gluc. Using blue spots as an indication of gene transfer and expression, we routinely obtained a few positive signals per infected explant. Although successful gene transfer seems to

occur as determined by the GUS staining, the major problems we have encountered has been elimination of *Agrobacterium* growth during the callus induction and shoot regeneration stages. So far, a combination of timentin and cefotaxime in the medium has been found to be the most effective treatment for repression of *Agrobacterium* growth. A few shoots have been obtained from these experiments, but no definite evidence of stable transformation has been obtained.

Transformation of wheat scutella using Agrobacterium.

Isolated scutella excised from the soft wheat cultivar AC Nanda have been used as explant material for genetic transformation by the *Agrobacterium* strain LBA4404/pTOK233. As found for *Agrobacterium* transformation of wheat inflorescences described above and barley (see Milestone 6), we have experienced severe problems with elimination of *Agrobacterium* after co-cultivation. A few plants surviving selection on media containing 40 mg/L hygromycin were obtained, but molecular analysis did not confirm stable transformation.

Milestone 6: Genetic transformation of selected barley genotypes with biolistics and Agrobacterium mediated gene delivery.

Particle bombardment of barley tissues

Some of the culture systems developed in Milestone 4 were used for transformation using particle bombardment and *Agrobacterium* to assess their effectiveness as target tissues for stable transformation. Two constructs carrying selectable marker genes were used. The construct pRC62 carries the *gus::nptII* fusion cassette driven by the rice actin promoter and the construct pUB16 carries the *bar* gene driven by the ubiquitin promoter (Milestone 1). These constructs were used individually for bombardment or either one was used for co-bombardment with the pRPL4 construct. The latter carries a disease-resistance gene driven by the rice actin promoter. In some experiments, the p35S-G1-1 construct carrying the GFP gene was used.

Determination of endogenous PPT-resistance levels in different barley cultivars

Some of our genetic transformation experiments were to include the *bar* gene (construct pUB16) as a selectable marker. The *bar* gene encodes resistance to the herbicide Basta (glufosinate ammonium), with the active ingredient L-PPT (phosphinothricin). Since cereals show great variation in sensitivity towards the herbicide, the L-PPT resistance levels for each explant tissue of wheat and barley must be determined before the explants can be used in transformation experiments. Prior to starting bombardment with the construct pUB16, an attempt was made at determining the endogenous PPT-resistance levels in barley genotypes.

A method that correlates the determined intrinsic resistance levels with the optimal L-PPT concentration to use in the regeneration media was published (Hänsch et al. 1998). We have successfully used the method and determined the optimal selection levels for the barley cultivars CDC Dawn, BT459 and HB335. Dose response curves for the L-PPT sensitive cultivar BT459 is shown in shown in Fig. 18.

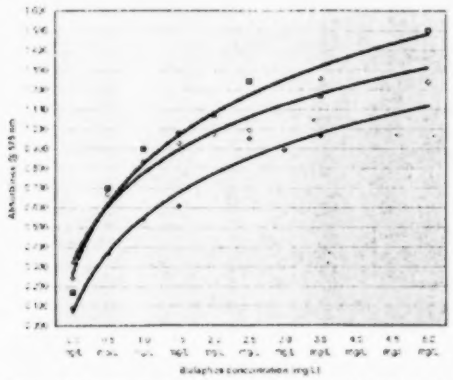


Fig. 18. Dose response curves obtained by chlorophenol red assays performed with the barley cultivar BT459. The data are from three independent experiments. According to Hänsch et al. (1998), the optimal concentration of bialaphos during selection corresponds to A_{575} value 0.5

Transformation of immature scutella of barley using particle bombardment

Procedures for isolating scutella and particle bombardment were as described in Milestones 3 and 5. However, for transformation experiments medium MB1 was supplemented with 100 mg/L of the antibiotic cefotaxime to avoid contamination.

Isolated scutella were left on the cefotaxime-containing medium in the dark for one day. Subsequently, the scutella were bombarded with plasmid DNA coated onto gold particles. After another day of incubation in the dark, the scutella were transferred to fresh medium MB1 containing no antibiotic. Cultures were incubated in the dark for three weeks at 24±1°C and thereafter exposed to low light intensity for one week. Calli induced from scutella were transferred to regeneration media. Once shoots started to appear (3-4 weeks), the calli were transferred to 0MS selection medium containing the appropriate selection agent, *i.e.*, either the antibiotic paramomycin or the herbicide L-PPT.

For transformation experiments conducted with genes encoding β–glucoronidase activity the success of gene delivery to target tissues was tested by GUS staining. GUS staining of samples of bombarded scutella was done 1-2 days post bombardment and results gave an indication of the level of transient GUS expression (Fig. 19).

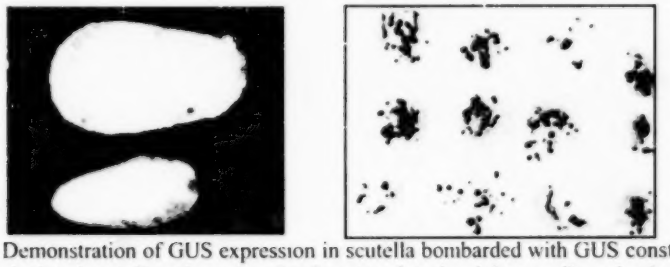


Fig.19. Demonstration of GUS expression in scutella bombarded with GUS construct. To the left is shown isolated scutella prior to bombardment and to the right scutella stained with X-Gluc two days post bombardment.

The results of the particle bombardment experiments are shown Table 16. Combining all the transformation experiments, a total of 4439 scutella were bombarded. A total of 325 regenerated plants survived the selection, an indication that they may be putatively transformed. For the control treatments (non-bombarded scutella), out of 464 explants only 27 plants survived selection. Among the surviving plants, 70 (1.8%) putatively transformed plants and three (0.6%) control plants attained maturity after being transferred to pots. The regeneration frequencies indicated that the levels of antibiotic and herbicide appear to have been effective for selection in most experiments. An exception is experiment AF2, wherein 24 non-transformed control plants survived

selection on L-PPT media. It may be that the explant material used in this experiment was exceptionally vigorous, thus tolerating a higher level of herbicide than in other experiments.

Table 16. Barley scutella transformation experiments by particle bombardment.

Exp.	Genotype	# explants Exp. / Control	Plasmids	# selected shoots Exp. / Control	# mature plants Exp. / Control
AF1	BT459	210 / 10	pUB16	0 / 0	-
AF2	BT459	360 / 30	pUB16	50 / 24	8 / 3
AF3	BT459	390 / 18	pUB16	28 / 0	
AF4	Dawn	420 / 30	pUB16	104 / 3	14 / 0
AF5	Dawn	300 / 30	pUB16	27 / 0	27 / 0
AF7	Dawn	420 / 30	pUB16	10 / 0	10 / 0
AF32	Dawn	249 / 22	pACTRPL4/pUB16	13 / 0	2 / 0
AF33	HB335	173 / 23	pACTRPL4/pUB16	11 / 0	1 / 0
AF36	TR159	304 / 30	pACTRPL4/pRC62	8 / 0	3 / 0
AFH1	Dawn	290 / 28	pACTRPL4/pRC62	11 / 0	1 / 0
AFH2	Dawn	90 / 28	pACTRPL4/pRC62	13 / 0	0
AFH3	Dawn	174 / 30	pACTRPL4/pRC62	12 / 0	0
AFH4	HB335	209 / 31	pACTRPL4/pRC62	28 / 0	4 / 0
AFH5	HB335	268 / 30	pACTRPL4/pRC62	0	0
AFH6	BT459	298 / 58	pACTRPL4/pRC62	0	0
DaLS1	Dawn	284 / 36	pACTRPL4/pRC62	10 / 0	0
Total		4439 / 464		325 / 27	70 / 3

Transformation experiments were also conducted with scutella using construct p35S-G1-1 carrying the GFP gene. These experiments were done to test if visual detection of GFP fluorescence could be used to identify and select transformed tissues of barley. This approach could allow development of transgenic plants free of antibiotic and/or herbicide resistance genes. Our results indicate that this may feasible (Fig. 20). Background fluorescence was adjusted using control treatments (non-bombarded calli), thereby allowing the detection of only sectors that have been transformed.

(a) (b)

Fig. 20. GFP expression in three week old calli produced from mature embryos bombarded with the p35S-G1-1 construct. Fluorescence was detected using a stereo microscope. (a) Control non-transformed explant. (b) Callus transformed with GFP construct.

Transformation of leaf-bases of barley using particle bombardment.

Two routes were taken for transformation of leaf-bases using biolistics, *viz*, bombardment of excised leaf-bases or bombardment of 7-10 days old leaf-base calli. Initially, it was observed that the same parameters used for bombardment of scutella were not applicable to leaf-bases. Using conditions for scutella bombardment, the leaf-bases turned brown a few days after bombardment and subsequently died. It was likely that the high pressure (1100 psi) used to accelerate the gold particles caused sufficient injury to the leaf-bases such that recovery was not possible. Reducing the shooting pressure by 40% circumvented this problem. Subsequent experiments with leaf-bases conducted at the lower pressure are summarized in Table 17. Bombarded leaf-base derived calli were used in experiments labeled Dalbc1, TRlbc1, HBlbc1 and Btlbc1. The remaining experiments consisted of excised leaf-bases shot soon after isolation.

Of a total of 2447 shot explants, 546 shoots survived selection and 390 (15.9%) plants attained maturity in pots. Compared to the scutella transformation data, the leaf-base system appears to be more effective. The number of scutellar explants bombarded was 4439, almost twice as much as the number of leaf-base explants. However, the number of mature plants produced from scutellar calli after selection was about 6 times lesser than the number produced from leaf-base calli.

Table 17. Barley leaf base transformation experiments by particle bombardment.

Exp.	*Genotype*	# explants Exp. / Control	Plasmids	# selected shoots Exp. / Control	# mature plants Exp. / Control
Dalbc1	Dawn	234 / 26	pACTRPL4/pUB16	31 / 3	34 / 0
TRlbc1	TR150	260 / 52	pACTRPL4/pUB16	171 / 31	154 / 3
HBlbc1	HB335	208 / 26	pACTRPL4/pRC62	32 / 3	34 / 0
BTlbc1	BT459	211 / 26	pACTRPL4/pRC62	73 / 13	32 / 0
Dalb1	Dawn	364 / 52	pACTRPL4/pRC62	28 / 0	6 / 0
Dalb2	Dawn	338 / 52	pACTRPL4/pRC62	54 / 4	34 / 1
Dalb3	Dawn	260 / 52	pACTRPL4/pRC62	51 / 2	22 / 2
HBlb2	HB335	182 / 26	pACTRPL4/pRC62	79 / 12	63 / 2
TRlb1	TR150	182 / 26	pACTRPL4/pRC62	10 / 1	4 / 0
BTlb1	BT459	182 / 26	pACTRPL4/pRC62	10 / 3	4 / 0
Btlblong	BT459	26 / 0	pACTRPL4/pRC62	7 / 0	3 / 0
Total		2447 / 364		546 / 72	390 / 8

Transformation of mature embryos of barley using particle bombardment

Bombardment parameters for mature embryos were similar to those used for scutella. Mature embryos were found to be more tolerant to injury during bombardment. Even during isolation, slight damage to the root primordia or the region surrounding root primordia did not affect regeneration response. However, care had to be taken not to inflict damage to the apical meristem region. All the experiments involved direct multiple shoot induction from the mature embryos after bombardment, with the exception of experiment DaMEc1, in which case calli were initiated after bombardment followed by regeneration. Table 18 shows the results obtained with mature embryos as target tissue for transformation. Out of a total of 939 mature embryo explants shot, 126 (13.4%) plants attained maturity. This regeneration frequency is proportionately comparable to the results obtained with leaf-bases.

Table 18. Barley mature embryo transformation experiments by particle bombardment.

Exp.	Genotype	# explants Exp. / Control	Plasmid(s)	# selected shoots Exp. / Control	# mature plants Exp. / Control
DaME		130 / 52	pRC62	19 / 8	8 / 2
HBME		182 / 26	pACTRPL4/pUB16	73 / 12	51 / 2
HBME1		133 / 26	pACTRPL4/pRC62	6 / 0	5
DaME2		104 / 26	pACTRPL4/pRC62	56 / 0	45
DaME3		156 / 78	pACTRPL4/pRC62	12 / 0	3
Damec1		234 / 26	pACTRPL4/pRC62	23 / 0	14
Total		939 / 234		189 / 20	126 / 4

Agrobacterium transformation of barley tissues.

Although *Agrobacterium*-mediated transformation of cereals has been reported, we found that published protocols were not reproducible. Therefore, we undertook several basic studies to optimize parameters for *Agrobacterium* transformation. One of the major problems was to eliminate the *Agrobacterium* cells after co-cultivation with the explants. Published levels of antibiotics for eliminating *Agrobacterium* were either too low or too high and in the latter case affected callus induction and regeneration. For example, the antibiotic, timentin, at low to high concentrations did not suppress growth of *Agrobacterium*, whereas the antibiotic cefotaxime was effective at suppressing growth of *Agrobacterium*. However, cefotaxime also affected explant, callus induction and regeneration. Scutella turned brown and produced tiny watery calli and subsequently died. A similar scenario was seen with leaf-base calli, except that the watery, non-embryogenic calli produced survived, but no regeneration was possible with cefotaxime in the medium. Upon transfer of calli to cefotaxime free media, *Agrobacterium* re-growth occurred. Timentin, although not toxic to leaf-bases, was ineffective at suppressing *Agrobacterium* re-growth. Moreover, transient GUS expression was not observed in any of the surviving calli after three weeks, in spite of using acetosyringone, as reported by several of the published procedures. Considering these obstacles, we set out to investigate ways of eliminating and/or preventing re-growth of *Agrobacterium* and

develop strategies for obtaining transient expression. The following approaches were considered:

(1). Effective concentration of antibiotics for suppression of *Agrobacterium* growth.

(2). Reduction in co-cultivation periods.

(3). Optimization of *Agrobacterium* cell density.

(4). Pre-treatment incubation medium for *Agrobacterium*.

To study the effective concentration of antibiotics for suppression of *Agrobacterium* growth after co-cultivation, filter paper disc assays were performed, using timentin and cefotaxime at different concentrations and *Agrobacterium* at different cell densities (Fig. 21 and 22). While effective concentrations of cefotaxime could be identified, timentin even at 500 mg/L did not suppress the growth of *Agrobacterium*. As expected, the higher cell densities required higher levels of cefotaxime for suppression of *Agrobacterium* growth. However, as mentioned earlier, such high levels of cefotaxime are also detrimental to embryogenic callus induction and regeneration. Therefore, for subsequent experiments with *Agrobacterium* we chose a lower level of cefotaxime in the media to suppress *Agrobacterium* growth to a great extent, without significantly affecting callus induction.

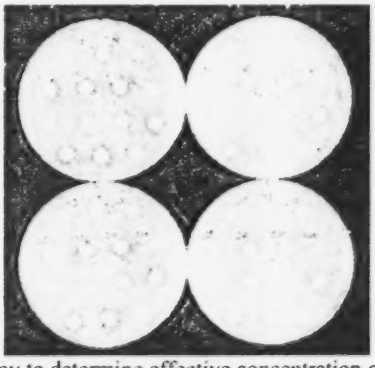


Fig. 21. Filter paper disc assay to determine effective concentration of antibiotic for the suppression of *Agrobacterium* growth. The diameter of clearing around the disc was measured to estimate the antibiotic's effect.

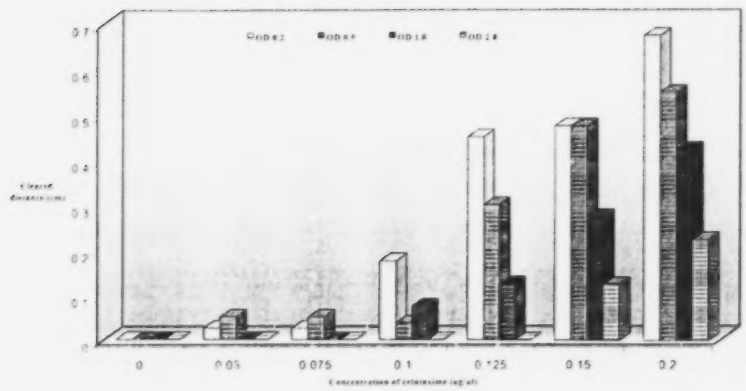


Fig. 22. Filter paper disc assay to determine efficient suppression of *Agrobacterium tumefaciens* of varying cell densities by increasing levels of cefotaxime.

Further manipulations with the co-cultivated explants included washing in sterile water and 100 mg/L cefotaxime. When re-growth of *Agrobacterium* occurred in cultures, the explants were given a rinse in sterile water and cefotaxime, and transferred to fresh medium. Although this approach appears to be effective to some extent, it is very laborious and not practical for large-scale experiments. Furthermore, opening of culture plates and rinsing explants increase the chance of introducing other contaminants. We did not pursue this approach further.

Other manipulations for successful *Agrobacterium* transformation included the pre-inoculation treatment. A time course study over a 24 h period was conducted to determine growth of *Agrobacterium* cells in liquid MS medium supplemented with either sucrose, glucose or maltose (Fig. 23). There was an increase in OD during the first three hours of incubation with all three sugars. However, glucose gave the best response, followed by maltose. Another increase was detected between six and nine hours and thereafter a plateau phase was reached, with not much of cell growth occurring. Since maltose is the optimal carbohydrate source in our tissue culture media, pre-inoculation incubation of *Agrobacterium* cells (OD in the range of 0.5 to 1.0) was done in liquid MS medium containing 3% maltose and 1% glucose. This was done as a conditioning of the *Agrobacterium* cells to our plant tissue media. The medium also contained 200 μM

acetosyringone. The incubation was carried out from 6-8 hours prior to inoculating explant material. With regards to co-cultivation period, the time was shortened to one day instead of two days. Also, prior to co-cultivation the explants were incubated with the *Agrobacterium* suspension for 30 min on a shaker.

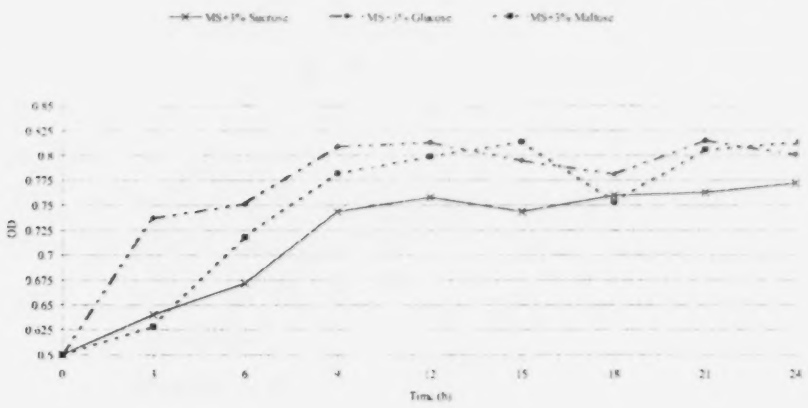


Fig. 23. OD values of *Agrobacterium* cultures over time when grown in MS media with different carbon sources.

Agrobacterium transformation of barley scutella

Taking all the above-mentioned optimized parameters into consideration, one week old scutella-derived calli were inoculated with *Agrobacterium*. Transient GUS expression was observed in CDC Dawn and CDC McGwire scutella-derived calli (Fig. 24). In subsequent experiments, when acetosyringone was omitted from the pre-incubation medium, transient GUS activity levels increased (Fig. 25). In two of the experiments more than 50% of the calli tested showed GUS activity when acetosyringone was excluded. This frequency is in sharp contrast to frequencies obtained (0-5%) when acetosyringone was used. Thus, it is possible that acetosyringone is not essential for induction of Vir genes when *Agrobacterium* infects barley tissues. Several studies with other plants have also reported improved transient GUS activity in the absence of acetosyringone.

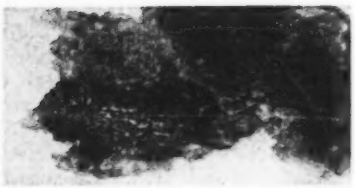


Fig. 24. Transient GUS expression in scutella-derived callus transformed with *Agrobacterium*.

(a) **(b)**

Fig. 25. Transient GUS expression from scutella-derived callus infected by *Agrobacterium* strain LBA4404/pTOK233. (a) Callus induced from scutellum of CDC Copeland; (b) Callus induced from scutella of CDC Dawn.

Re-growth of *Agrobacterium* cells after co-cultivation with explant tissues has continued to be a problem. Our strategy of using cefotaxime at a low level (25 mg/L) in the media has been found to be insufficient for the complete elimination of *Agrobacterium*. This concentration is effective for a short period of time (1-2 weeks) and thereafter *Agrobacterium* re-growth occurs. As mentioned earlier, higher levels of cefotaxime invariably affected callus induction and plant regeneration. We have also tested a biocide, Plant Preservative Mixture (PPM), which has been reported not to be toxic to plant tissues and is capable of suppressing both fungal and bacterial growth, including *Agrobacterium*. Although phytotoxic effects on explants or calli were not observed, re-growth of *Agrobacterium* occurred after about three weeks, even at a high level of PPM (5 ml/L).

Agrobacterium transformation of barley leaf-bases.

The parameters used for *Agrobacterium* transformation of scutella were also applied to leaf-base explants. Transient GUS expression has been observed with CDC Dawn and BT459 leaf-bases (Fig. 26 a and b).

(a) (b)

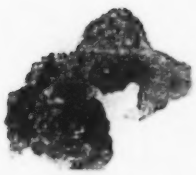


Fig. 26. GUS expression in leaf bases transformed with *Agrobacterium tumefaciens* strain LBA4404/pTOK233. (a) Leaf-base derived callus showing GUS expression. (b). Leaf base cells showing spots patches of GUS activity

Two approaches of transformation with *Agrobacterium* have been tried and both have been successful. In the first approach, three-day old seedlings were injected with a suspension of *Agrobacterium* cells in the meristematic region, *i.e.,* at the base of the shoot. After one day incubation on moist filter paper, leaf-bases were excised and transferred to callus induction medium. In the second approach, leaf-bases were excised and transferred to callus induction medium. After 7-10 days, calli were co-cultivated with *Agrobacterium* for 1-2 days.

Agrobacterium transformation of barley mature embryos.

Two experiments were conducted to test if mature embryos could be transformed by *Agrobacterium*. Since the meristematic tissue is embedded within primary rudimentary leaves, the coleoptile and the shoot sheath, targeting the *Agrobacterium* cells to that region is difficult. We have used controlled enzymatic digestion on isolated mature embryos. Mature embryos were treated with a combination of macerase and cellulase for 30 min to cause enough damage to the outer layers of cells. The explants were rinsed in sterile water several times and incubated with *Agrobacterium* cell suspensions for 30 min. Co-cultivation was done for one day and explants were transferred to PPM containing medium for multiple shoot induction. In spite of recurring growth of *Agrobacterium*, several putative transformants that survived selection, were transferred to pots and grown to maturity.

Milestone 7: Greenhouse evaluation of wheat and barley transgenics

As described in Milestone 5, a total of 608 regenerated wheat plants were obtained from transformation experiments using particle bombardment and various explants. Leaves of the generated primary transformants or their progeny have been collected, freeze-dried and stored at –70C for molecular analysis. DNA was extracted from most of the collected leaf samples and about 1/3 of these have been analysed by PCR.

Screening of wheat lines transformed with Sbe2 anti-sense constructs.

About 50 putative wheat transformants derived from scutella or inflorescence bombarded with anti-sense *Sbe2* constructs have been screened for the presence of transgenes. This screening was done by PCR analysis using primers specific for the anti-sense *Sbe2* or the selectable marker gene and DNA extracted from leaves. An example of such a screening for anti-sense *Sbe2*-positive plants is shown in Fig. 27. All the 16 plants positive for anti-sense *Sbe2* construct were also found to carry the selectable marker gene.

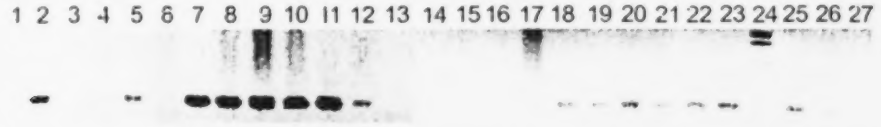


Fig 27. PCR analysis of plants regenerated from particle bombardment of Fielder scutella and Karma inflorescence. Reactions from samples lacking DNA template are shown in lanes 1 and 14. Lanes 2 – 11 contain reaction of plants regenerated from Fielder scutella bombarded with plasmids of αBE23 + pRC62 or pKA8. Plants regenerated from Karma inflorescence bombarded with plasmids of αBE23 + pKA8 are analysed in lanes 12, 18 – 22 and 25 – 27. Reactions of non-transformed Fielder (lanes 13 and 15) and non-transformed Karma (lanes 16 and 17) are also shown. Lane 23 contains reaction with plasmid αBE23 used as positive control. Migration of DNA molecular weight markers is shown in lane 24. Reactions producing a ~200 bp fragment are positive for the anti-sense *Sbe*2b transgene.

Screening of wheat lines transformed with Fusarium resistance gene.

Six plants were regenerated from three transformation experiments with durum wheat scutalla (cultivar Plenty) bombarded with pBARGUS and pActRPL4 (experiments P37, P39 and P40 in Table 13). PCR screening of DNA extracted from the primary transformants revealed that five of the plants carried at least one intact copy of the

Fusarium resistance gene encoded by construct pActRPL4 (Fig. 28). Leaves of transgenic and non-transformed lines were pasted with 0.004% L-PPT to test for BAR activity. After one week's treatment, pasted leaves of four of the transgenic lines were still green, whereas treated leaves of parent line or non-transformed lines had turned brown. One transgenic line carrying the BAR gene did not show any resistance to L-PPT, probably due to gene silencing.

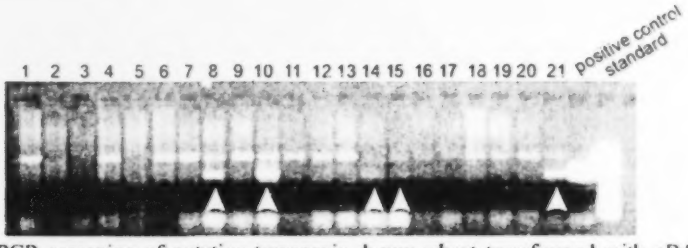


Fig. 28. PCR screening of putative transgenic durum wheat transformed with pBARGUS and pActRPL4. Arrows indicate Plenty lines carrying BAR gene.

Screening of wheat lines transformed with Sbe1c anti-sense construct.

Transformation experiments AF70 and AF71 (see Milestone 5) using Fielder (soft white spring wheat) scutella bombarded with expression cassettes α-Sbe1c and pKA8 produced 31 plants that survived the Paramomycin selection step. Total DNA was extracted from leaves of the regenerated plants and analyzed by PCR for the presence of transgenes (Fig. 29). The results of the PCR analysis is summarized in Table 19 showing that 24 of the 31 analysed plants was positive for NPTII gene. Fifteen transgenic plants were found to PCR-positive for both expression cassettes and nine plants were only positive for the selectable. Seven of the plants appeared from to have survived selection without being transformed with the *nptII* expression cassette.

Fig. 27. PCR screening of 22 primary wheat transformants obtained from transformation experiments AF70 and AF71 performed with Fielder (FD) scutella and pKA8+α-*Sbe1c* constructs. Primers specific for *nptII* were used in the PCR reactions. Control samples were done with parent DNA (FD) and no DNA template (-).

Mendelian inheritance of transgenes.

Six seeds from each of the 12 independent transgenic wheat plants carrying pKA8 and the anti-sense starch branching enzyme construct, α-*SbeIc*, were planted in the greenhouse and grown to maturity. Several control lines including the parent line Fielder (four plants), progeny of one line carrying only pKA8 sequences and four "escape lines" (lines that survived selection but lacked transgene) were also grown. All seeds germinated at normal rate and produced plantlets with no obvious abnormalities.

Leaves of the developing plants were collected and analyzed by PCR for inheritance of α-*SbeIc* and pKA8 sequences. An example of such an analysis is shown in Fig. 30 and a summary of the PCR data is presented in Table 19. The analysis showed that the transgenes were inherited in a Mendelian fashion for 11 out of the 12 lines studied. The segregation ratio for progeny of line 70:13 was found to be closer to 1:1 than the expected 3:1. All of the transgenic offsprings were found to co-inherit both introduced genes (*nptII* and α-*SbeIc*).

Fig. 28. PCR analysis of progeny of transgenic R0 plants using primers specific for the α-*SbeIc* expression construct. Four off-springs of six transgenic plants were analyzed. Analysis of the parent line, Fielder (FD), sample without DNA sample (-) and positive control sample (α-*SbeIc* construct) are also shown.

Table 19. PCR analysis of the R1 generation of transgenic wheat lines.

Generation	Transgene(s)	FD	AF70 series																							AF71 series						
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	1	2	3	4	5	6	7
R0	*nptII*	-	+	+	-	+	+	+	+	(+)	+	+	-	+	+	(+)	+	+	(+)	(+)	-	-	+	-	+	-	+	+	+	+	+	+	-
R0	α-*SbeIc*	-	-	-	-	+	+	+	+	-	+	+	-	+	+	-	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	+	-
R1	*nptII*+α-*SbeIc**					6/8	6/8	5/6	7/8		7/8	6/8		6/8	3/8		6/8	6/8		ND			ND		7/8					7/8		ND	

* both transgenes were co-inherited. Ratio given indicate # R1 plants carrying transgenes / # R1 plants lacking transgenes.

ND = not determined.

Southern blot analysis of 12 of the PCR-positive progenies confirmed that the α-*Sbe1c* and *nptII* (data not shown) sequences were integrated into the genome (Fig. 31). One of the PCR-negative plants was found to carry a rearranged or truncated version of α-*Sbe1c* expression cassette (plant 71:3:2:1).

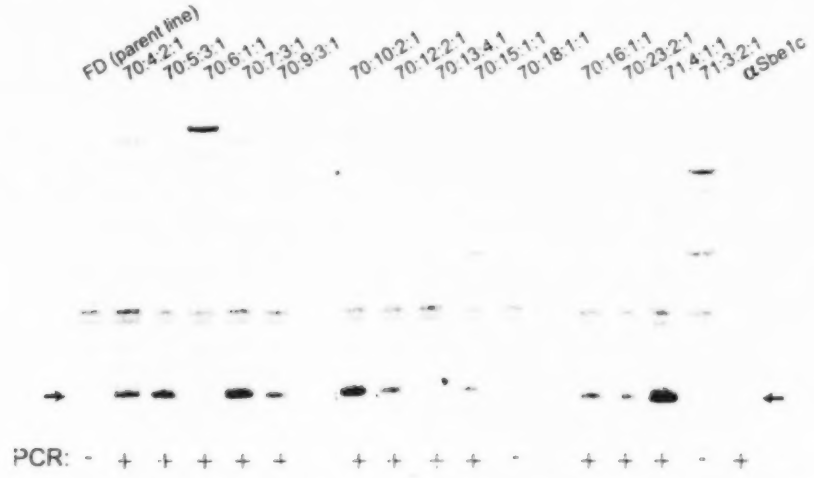


Fig. 29. Southern blot analysis of HindIII-digested genomic DNA extracted from R1 plants of Fielder (FD) transformed with anti-sense *Sbe1c* construct (α-*Sbe1c*) and pKA8. Hybridization between the *Sbe1c* probe and FD DNA gave signals to 10 HindIII fragments carrying endogenous *Sbe1* and *Sbe1c* genes. The migration of intact inserted transgene fragment derived from anti-sense *Sbe1c* plasmid is indicated by horizontal arrows.

Delayed heading in transgenic anti-sense Sbe1c wheat lines.

When the progeny of α-*Sbe1c* plants reached the heading stage, a variation in height and date of anthesis was noted among tissue culture-derived plants (Fig. 30). Most of the progeny of the transgenic plants showed delayed heading, and for some lines, anthesis occurred up to three weeks later than for the parent line, Fielder. The difference in height or the delayed onset of anthesis could not be 100% correlated with the presence of transgene(s). A few progeny that did not carry the transgenes, as determined by the PCR analysis, showed about the same heading date as transgenic progeny derived from the same mother plant. However, it is possible that these few lines carried rearranged or truncated form α-*Sbe1c* sequences, that were not amplified in the PCR reaction.

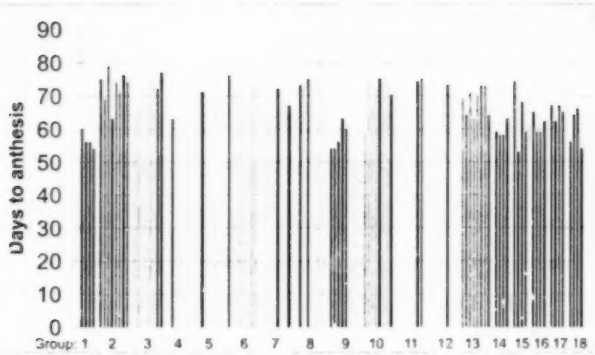


Fig. 30. Days to anthesis in transgenic and non-transgenic wheat grown under greenhouse conditions. Group 1 represents plants of the parent, Fielder, and groups 2 to 13 are represented by progeny of 12 primary transformants. Colored bars indicate plants carrying the α-*SbeIc* construct and black bars represent plants lacking transgene. Group 14 contain progeny of a line transformed only with pKA8 and groups 15 to 18 are progeny of non-transformed (escape) lines surviving the selection step of the transformation experiments.

Table 20. Heading time in transgenic wheat lines

	R1 generation			R2 generation		
Line	Seed #	Transgenic	Days to anthesis	Seed #	Transgenic	Days to anthesis
Fielder (parent)	1	No	58	1	No	49
70-12	1	Yes	71	1	Yes	72
	2	No	73	2	Yes	83
	3	Yes	73	3	No	65
	4	No	75	4	Yes	73
	5	Yes	72	5	Yes	67
	6	Yes	74			
	7	Yes	81			
	8	Yes	69			
70-16	1	Yes	69	1	Yes	65
	2	Yes	72	2	Yes	72
	3	Yes	63	3	Yes	74
	4	Yes	72	4	Yes	81
	5	Yes	74	5	Yes	73
	6	No	74			
	7	No	75			
	8	Yes	79			

To study if delayed heading was an inherited trait, the heading time for two transgenic lines was determined for the next generation. This analysis showed that the R2 generation plants had a similar heading time as R1 plants (Table 20), and thus, the late flowering phenotype was an inherited trait and not induced by the tissue culture.

Altered starch granule distribution in transgenic wheat lines.

Analysis of starch granules extracted from endosperm of mature seeds produced by some of the primary transformants carrying the anti-sense *Sbe1c* construct suggested that the distribution of small (<10 um) and large (>10um) granules was altered. To determine if this altered granule size was an inherited trait, and not induced by tissue culture conditions, starch granule size distribution was determined for progeny of the primary transformants. This analysis also showed that certain transgenic lines carrying the anti-sense *Sbe1c* construct had an increased large/small granule ratio as compared to the parent line, Fielder (Table 21). Thus, our analysis suggested that reduced expression of *Sbe1c* in wheat affects both the heading time, as well as starch granule size distribution. Further molecular and biochemical analyses are required to determine the exact action of SBEIc in wheat endosperm.

Table 21. Starch granule size distribution in regenerated plants.

Transgenic plants:				Non-transgenic plants			
nptII-positive:		*nptII+uidA* positive:		"Escapes":		Parent (Fielder):	
Plant #	% B	Plant #	% B	Plant #	% B	Plant #	% B
70:1	78	70:5	69; 66; 34; 60; 49	70:3	78	1	80
70:2	73	70:9	72; 78; 43; 51; 73; 57; 64; 75	70:11	76	2	81
70:14	79	70:16	60; 67; 57	70:19	80	3	87
70:18?	77	70:21	71	70:20	82	4	77
71:1	79	71:3	69	70:22	76		
71:2	80	71:4	69; 66; 82	70:24	84		
71:5	72	71:6	70	71:7	78		

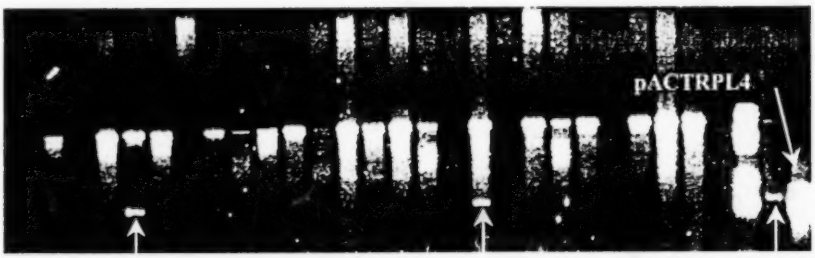
Characterization of putative barley transgenic plants.

Over 600 primary barley transformants have been generated during the course of this project using the different approaches. All were grown to maturity in the greenhouses or growth chambers. Regenerated plants appeared normal overall as compared to donor plants, with the exception of a few plants that produced few tillers, showed short stature and matured early. Leaves, if enough were available, were collected from these primary transformants and freeze-dried for large-scale DNA extraction for DNA blot analyses. Mini-DNA extractions were also performed for use in PCR assays. Some of the PCR positive primary barley transformants were advanced to R1 generation and appeared

normal. PCR assays have been done on most of them and DNA blot analyses on some of them.

Many of the primary barley transformants have been assayed by PCR for the genes of interest. Fig. 31 shows PCR profiles for barley transformants carrying a Fusarium-resistance gene and/or the selectable marker gene, *bar*.

(a)



(b)

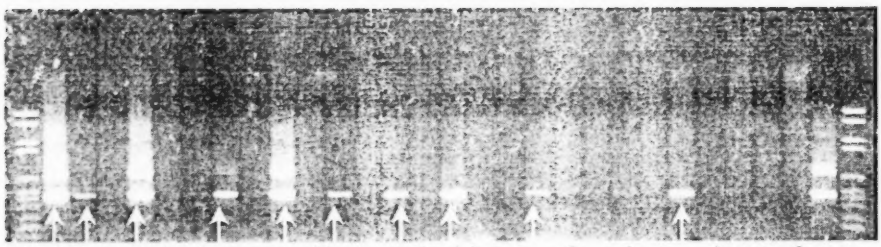


Fig. 31. PCR analysis of regenerated barley derived from transformation experiments of mature embryo cultures.
(a). Analysis for the presence of the Fusarium-resistant gene fragment. Arrows indicate band corresponding to expected fragment.
(b). Analysis for the presence of the BAR gene fragment. Arrows indicate band corresponding to expected fragment.

Many of the transformants have been produced by co-bombardment of the Fusarium-resistance gene construct and the GUS::NPTII fusion construct. A multiplex PCR assay has been developed to assay for the GUS and the NPTII genes simultaneously, thereby reducing the time and number of reactions to be conducted (Fig. 32).

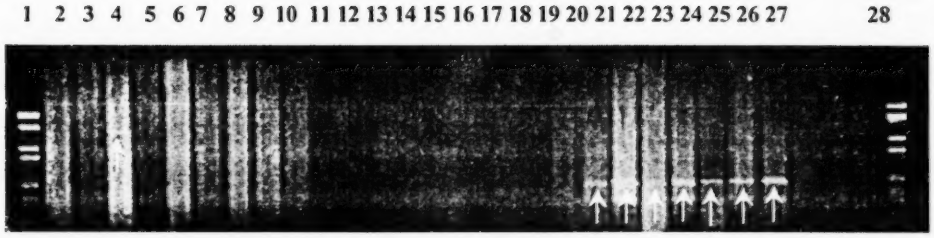
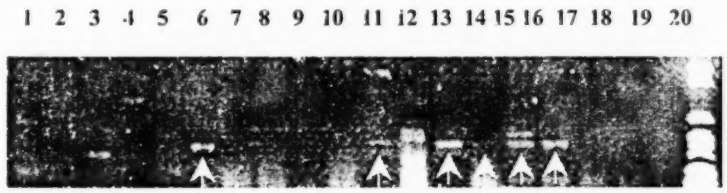


Fig. 32 Multiplex PCR analysis for the GUS and NPTII genes on primary transformants generated by particle bombardment. Lanes 1 and 28: Molecular weight marker; Lanes 2-26: putative transformants; Lane: 27: Non-transformed control; Lane 2: transformant positive for the GUS gene; Lanes 20-26: transformants positive for the NPTII gene. Arrows indicate bands of interest.

Barley R1 plants derived from PCR positive primary transformants (Fig. 31a and b) were grown in a growth chamber and PCR assays were performed on these plants. Fig. 33 shows the PCR profiles for the BAR and the Fusarium resistance gene.

(a)



(b)

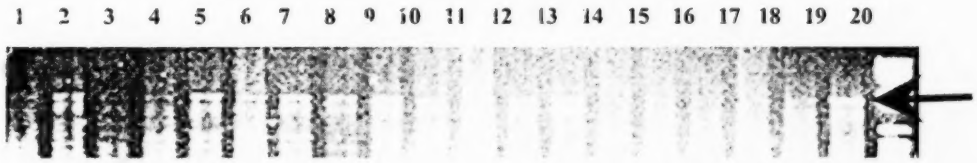


Fig. 33 PCR analysis of barley plants. (a) Identification of plants carrying BAR gene. Lanes 1-18: R1 progeny of putative transformants; Lane 19: non-transformed control; Lane 20: molecular weight marker. Arrows indicate migration of BAR gene fragment.
(b) Identification of plants carrying Fusarium resistance gene. Lanes 1, 18 and 20: R0 transformants, Lanes 2-13: R1 plants; Lanes 14, 15 and 16: 100 ng of barley DNA spiked with 1, 0.1 and 0.01 pg of pRPL4 plasmid carrying the Fusarium resistance gene; Lane 17: non-transformed control. Arrow indicates migration of Fusarium-resistance gene fragment.

Milestone 8: Molecular / cytogenetic characterization of wheat and barley transgenics to compare integration of inserted genes into host genomes produced with biolistics or *Agrobacterium*-mediated gene delivery.

A comparison of transgenic lines obtained through particle bombardment and *Agrobacterium*-mediated transformation was not done, due to lack of confirmed *Agrobacterium* transformed wheat and barley lines. Analysis data of wheat and barley transgenic lines obtained from particle bombardment are presented in Milestones 6 and 7.

Milestone 9: Field-testing of barley trangenics.

Progeny of seven barley lines transformed with the pUB16 construct (Milestone 1) were field-tested during the growing season of 2000 (Fig. 34) after obtaining approval from the Canadian Food Inspection Agency. The tested plants were derived from four transformed feed (CDC Dawn) and three six-row malting (BT459) lines, all independently transformed. The seeds were planted in rows of 50 and spray-tested at the three-leaf stage for resistance to the herbicide Liberty Link™. However, spraying with normal dose (1.6 L/acre) and low dose (0.8 L/acre) did not reveal any herbicide resistance for any of the progeny of the transgenic lines after one week of spraying..

The lack of *bar* expression could be due to gene silencing or instability of the transgene. To test if the transgene was present in the tested lines, two of the non-sprayed plants from each transgenic line were analyzed by PCR assays. Twenty of the analyzed samples representing progeny of six of the seven independent transformants were found to be positive for the BAR gene (Fig. 35). PCR assays were also performed on DNA samples collected from plants prior to herbicide treatment. The PCR profile for six of the herbicide-sensitive plants showed presence of the resistance gene, b*ar* (Fig. 36). Although DNA blot analyses have not confirmed the presence of the gene(s) in all the PCR positive lines from the field test, the data strongly suggested that the lack of *bar* expression in most plants tested was not due to lack of transgene. Probably, gene silencing was the main reason for herbicide sensitivity.

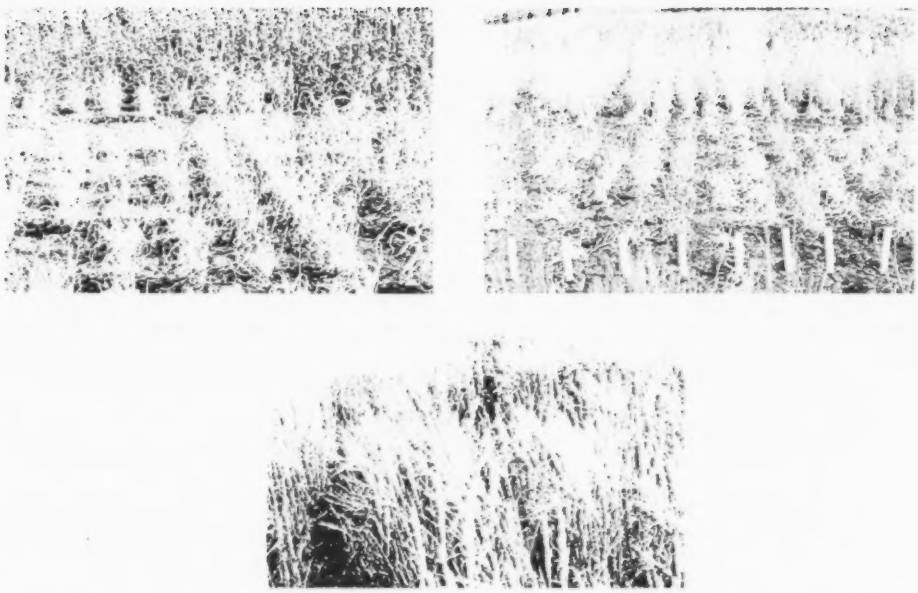


Fig. 34 Test of herbicide-resistance in transgenic barley lines carrying the pUB16 construct. (a) Three weeks old plants sprayed with Liberty at a rate of 1.6 L/acre on ten plants in each row. (b) Second set of ten plants sprayed (six weeks old) with lower rate of Liberty at 0.81 L/acre. (c) Mature non-sprayed transgenic plants.

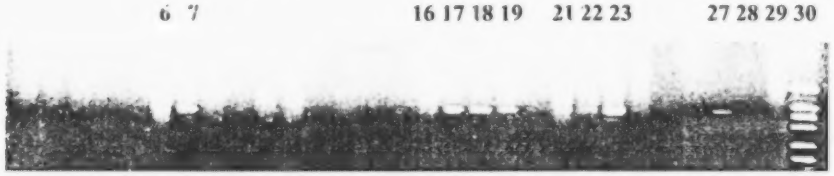


Fig. 35 Gel analysis of PCR reactions performed on leaf material of field-tested transgenic barley lines. Reactions in lanes Samples 1 to xx are from plants that were not sprayed with herbicide. Lanes 6, 7, 16, 17, 18, 19, 21, 22 and 23: BAR positive plants; Lane 27: 0.01 pg of plasmid pUB16 carrying the BAR gene. Lane 28: negative control (no DNA); Lane 29: non-transformed control; Lane 30: molecular weight marker.

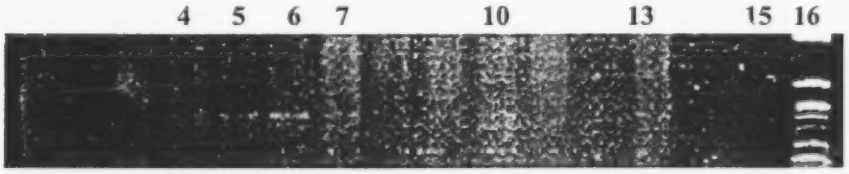


Fig. 36 PCR analysis of DNA samples from plants that were sensitive to Liberty Link. Bar-positive samples were identified in lanes 4, 5, 6, 7, 10 and 13. Non-transformed control plant was analysed in lane 15.Lane 16: molecular weight marker.

Young leaves of transgenic lines were assayed to measure the phosphinotricin acetyl transferase (PAT) activity encoded by the BAR gene. However, the results from the PAT assay were inconclusive. Another assay that is based on differences in ammonium evolution from PAT-positive and PAT-negative plants was conducted. Control experiments with wheat lines confirmed that non-transformed plants produce more ammonia than transgenic lines expressing the BAR gene. This assay is simple and gives qualitative and quantitative measurements of ammonium evolution as a measure of PAT activity. However, analysis of the barley lines did not reveal any PAT activity from the transgenic lines.

The transgenic plants derived from the non-sprayed part of the rows appeared to be normal and no differences in heading dates, height and maturity were evident. Thousand kernel weight for all transgenic lines and non-transformed control plants were measured and showed some variation (Fig. 37). Most of the lines either showed higher or about the same thousand kernel weights as the non-transformed lines.

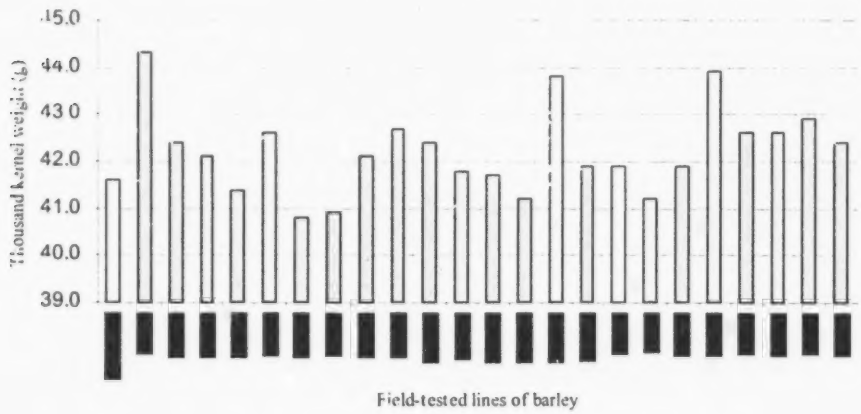


Fig 37. Thousand kernel weights determined for transgenic lines derived from CDC Dawn and BT459 and field-tested during the growing season of 2000. White bars indicate non-transformed plants of the two genotypes.

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